DETECTION OF MUTATIONS IN THE FACTOR VIII GENE BY mRNA ANALYSIS

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Hemophilia A is an X-linked bleeding disorder caused by deleterious mutations in the coagulation factor VIII gene. The factor VIII gene located at Xq28 and spanned 186 kb, is composed of 26 exons and transcribes mRNA with the length of about 9 kb. The molecular defects of factor VIII gene causing hemophilia A are heterogeneous, giving rise to clinical heterogeneity. The characterization of pathogenic mutations of the factor VIII gene allows the recognition of new mechanisms of functional disturbances of factor VIII. The molecular characterization of mutations in Thai hemophilia A patients in this study was carried out by the methods of reverse transcription-polymerase chain reaction (RT-PCR), single strand conformation polymorphism (SSCP), and direct DNA sequencing. The RT-PCR method for amplification across the inversion break-point region was used to screen the factor VIII gene inversion, which separates exons 1-22 from exons 23-26 and is found to be a common cause of severe hemophilia A. A combination of long RT-PCR of the factor VIII mRNA and PCR of factor VIII genomic DNA were used to isolate the factor VIII sequence for screening small molecular defects. The entire factor VIII cDNA sequences were amplified and fractionated into seven overlapping fragments by nested PCR. The exon 14 was amplified into two overlapping fragments either by using long cDNA or genomic DNA as a template. The fragments were digested with appropriate restriction enzymes to generate the size suitable for SSCP. DNA sequencing was used to identify mutations detected by the SSCP screening. Once the mutations were identified, the allele-specific amplification (ASA) was developed and used for analysis in all available members of the family to confirm the identification and examine the carrier status.

RT-PCR analysis for the gene inversion of 11 samples from severe hemophilia A patients revealed the discontinuity of RNA when a PCR on reverse transcribed RNA was performed across exons 22-23, demonstrating the presence of factor VIII gene inversion. Ten of these patients have been investigated to have gene inversions by Southern-blot hybridization. The RT-PCR method developed is useful for screening factor VIII gene inversion. cDNA analysis of 18 hemophilia A patients revealed the skipping of total 154 bp of exon 15 combined with the absence of the first 47 bp of exon 16 in one patient due to the G to T transversion at the donor splice site of intron 15 and the activation of a cryptic acceptor splice site within exon 16. The SSCP analysis was performed in 8 hemophilia A patients from 6 families. The mobility shifts were observed in the same cDNA fragment in 2 patients of the same family. From DNA sequencing, it was found that there was a nucleotide substitution at codon 233, ACA>ATA, in exon 6 of the factor VIII gene. This mutation resulted in a novel missense mutation (T233I) in the A1 domain. These results suggest that carrier status in members of both families could be detected by the ASA technique.

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ฮีโมฟีเลีย-เอ (hemophilia A) เป็นโรคทางพันธุกรรมที่ทำให้เกิดภาวะเลือดออกง่ายผิดปรกติแต่หยุดยาก เกิดขึ้นเนื่องจากความผิดปรกติของยีนแฟคเตอร์แปด (factor VIII gene) ยีนแฟคเตอร์แปดเป็นยีนที่มีขนาดใหญ่ (186 กิโลเบส) มีตำแหน่งอยู่ที่ส่วนปลายของโครโมโซม เอ็กซ์ (Xq28) ประกอบด้วย 26 เอ็กซอน และสังเคราะห์ เอ็ม-อาร์เอ็นเอขนาด 9 กิโลเบส ความผิดปรกติของยีนแฟคเตอร์แปดที่ทำให้เกิดโรคฮีโมฟีเลียมีความหลากหลาย ส่งผลให้มีความแตกต่างในความรุนแรงของโรค การศึกษารายละเอียดมิวเตชั่นของยีนแฟคเตอร์แปดซึ่งทำให้เกิดโรค จะนำไปสู่การเรียนรู้กลไกใหม่ๆ ที่กระทบต่อการทำงานของโปรตีนแฟคเตอร์แปด งานวิจัยนี้ได้ทำการศึกษามิวเตชั่นในผู้ป่วยไทยที่เป็นโรคฮีโมฟีเลีย-เอ โดยวิธี reverse transcription-polymerase chain reaction (RT-PCR), single strand conformation polymorphism (SSCP) และการวิเคราะห์ลำดับนิวคลีโอไทด์ของดีเอ็นเอ (DNA sequencing) วิธี RT-PCR ซึ่งเป็นการทำ amplification บริเวณที่เกิดอินเวอร์ชั่น นำมาใช้ตรวจมิวเตชั่นชนิดยีนอินเวอร์ชั่น (gene inversion) ซึ่งแยกเอ็กซอน 1-22 ออกจาก เอ็กซอน 23-26 และพบได้บ่อยในผู้ป่วยฮีโมฟีเลีย-เอ ชนิดรุนแรง วิธี long RT-PCR โดยการสังเคราะห์ซี-ดีเอ็นเอ จากเอ็ม-อาร์เอ็นเอของยีนแฟคเตอร์แปด และการทำ PCR ของจีโนมิค-ดีเอ็นเอ ได้นำมาใช้เพื่อแยกลำดับนิวคลีโอไทด์ของยีนแฟคเตอร์แปดเมื่อตรวจกรองมิวเตชั่นอื่นๆที่มีการเปลี่ยนแปลงเพียงเล็กน้อยภายในยีน ซี-ดีเอ็นเอของแฟคเตอร์แปดยีนทั้งหมด ถูกแบ่งเป็น 7 ชิ้น ที่เหลื่อมกันโดยวิธี nested PCR ส่วนบริเวณเอ็กซอน 14 ถูกแบ่งเป็น 2 ชิ้นที่เหลื่อมกันโดยการใช้ ซี-ดีเอ็นเอ หรือ จีโนมิค-ดีเอ็นเอ ชิ้นดีเอ็นเอเหล่านี้ จะถูกนำมาตัดเพื่อให้มีขนาดที่เหมาะสมด้วยเอ็นไซม์ตัดจำเพาะก่อนที่จะนำไปศึกษาด้วยวิธี SSCP และวิเคราะห์มิวเตชั่นด้วยวิธีวิเคราะห์ลำดับนิวคลีโอไทด์ เมื่อพบมิวเตชั่น จะทำการพัฒนาวิธี allele specific amplification (ASA) สำหรับตรวจมิวเตชั่นเพื่อหาผู้ที่เป็นพาหะในครอบครัว

จากการศึกษายีนอินเวอร์ชั่นด้วยวิธี RT-PCR ในผู้ป่วย 11 ราย พบว่าผู้ป่วยทั้งหมดมี เอ็กซอน 22 และ 23 ที่แยกจากกัน แสดงว่ามีอินเวอร์ชั่นของยีนแฟคเตอร์แปด ผู้ป่วย 10 ใน 11 ราย เคยตรวจมาก่อนว่ามียีนอินเวอร์ชั่นด้วยวิธี Southern-blot hybridization วิธี RT-PCR ที่พัฒนาขึ้นนี้สามารถใช้ตรวจกรองมิวเตชั่นชนิดอินเวอร์ชั่นของยีนแฟคเตอร์แปดได้ การศึกษาซี-ดีเอ็นเอในผู้ป่วย 18 คน พบว่ามีผู้ป่วย 1 คน ที่มีขนาดของชิ้นดีเอ็นเอสั้นกว่าปรกติและจากการวิเคราะห์ลำดับนิวคลีโอไทด์ พบว่ามีการขาดหายไปของเอ็กซอน 15 ทั้งหมด (154 bp) และส่วนหนึ่งของเอ็กซอน 16 (47 bp) ซึ่งเกิดจากการมีมิวเตชั่นที่บริเวณ donor splice site ร่วมกับการกระตุ้น cryptic acceptor splice site ภายในเอ็กซอน 16 วิธี SSCP ได้ถูกนำมาหามิวเตชั่นในผู้ป่วย 8 คน จาก 6 ครอบครัว จากการศึกษาพบว่าผู้ป่วย 2 คน ที่มาจากครอบครัวเดียวกันมีการเคลื่อนที่ผิดปรกติของดีเอ็นเอชิ้นเดียวกัน จากผลการวิเคราะห์ลำดับนิวคลีโอไทด์ พบว่ามีการแทนที่ของนิวคลีโอไทด์หนึ่งตัวที่ codon 233 จาก ACA>ATA ในเอ็กซอน 6 ของยีนแฟคเตอร์แปด ทำให้เกิดการเปลี่ยนของกรดอมิโนที่ตำแหน่ง 233 (T233I) ในบริเวณโดเมน A1 มิวเตชั่นชนิดนี้ยังไม่เคยมีรายงานมาก่อน การตรวจผู้ที่เป็นพาหะของฮีโมฟีเลีย-เอในทั้ง 2 ครอบครัวสามารถทำได้ด้วยวิธี ASA ที่ได้ทำการพัฒนาขึ้น

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LIST OF ABBREVIATIONS

dATP	=	deoxyadenosine-5'-triphosphate
dCTP	=	deoxycytidine-5'-triphosphate
dGTP	=	deoxyguanosine-5'-triphosphate
dTTP	=	deoxythymidine-5'-triphosphate
dNTP	=	dATP, dCTP, dGTP and dTTP
DEPC	=	diethylpyrocarbonate
DTT	=	dithiothreitol
EDTA	=	ethylenediamine tetraacetic acid
FVIII	=	coagulation factor VIII
FVIII:C	=	coagulation factor VIII activity
IVS	=	intervening sequence
kDa	=	kilodalton (s)
LB	=	Luria-Bertani (medium)
OD	=	optical density
PAGE	=	polyacrylamide gel electrophoresis
PBS	=	phosphate buffer saline
PEG	=	polyethylene glycol
RNase	=	ribonuclease
rpm	=	revolution per minute
SDS	=	Sodium dodecylsulfate
TEMED	=	N,N,N',N'-tetramethyl-ethylenediamine
w/w	=	weight/weight

CHAPTER I

INTRODUCTION

The blood coagulation mechanism is composed of a series of linked proteolytic reactions (1). Each reaction involves enzymatic conversion of an inactive precursor to an activated form, which in turn acts as an enzyme in the activation of the succeeding factor. These transformations result in the generation of thrombin, which acts on fibrinogen to finally produce fibrin, an essential component of the hemostatic plug. Factor VIII is one of the cofactor in the coagulation cascade for the activation of factor X by activated factor IX (FIXa) in the presence of phospholipid and calcium ion (2).

Mutations in the factor VIII gene cause hemophilia A, an X-linked recessive bleeding disorder, which affects 1 in 5000 males and very rarely in females. Located in the most distal band (Xq 28) of the long arm of the X chromosome (3, 4), the factor VIII gene is a large gene (186 kb) composed of 26 exons and transcribing a 9-kb mRNA which encodes a precursor protein of 2,351 amino acids (5). Factor VIII protein comprises a leader peptide, three A domains, a B domain containing several potential N-glycosylation sites, and two C domains (A1-A2-B-A3-C1-C2) (6).

A large inversion causing disruption of the factor VIII gene, due to homologous recombination between the intragenic F8A gene within intron 22 of the factor VIII gene and one of the two telomeric copies of it (7), has been shown in 40-50% of severe hemophilia A cases. Supplementary to the frequent rearrangement at intron 22, a wide spectrum of different mutations (point mutations, insertions and deletions) have already been reported (8).

Screening of all possible mutations in the factor VIII gene is hampered by its large size and high frequency of *de novo* mutations that results in different mutations in unrelated affected individuals (9). Taking advantage of the polymerase chain reaction (PCR) and several screening methods, for examples single strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), and chemical cleavage of mismatch (CCM), analysis of mutations in a large gene like the factor VIII gene is possible. Characterization of mutations will help to identify amino acids or regions with essential functional or structural properties and therewith clarify the mechanism of molecular pathogenesis. Mutations of the factor VIII gene in Thai hemophilia A patients have been systematically analyzed with the aim to understand the molecular defects and to develop direct methods for carrier detection and prenatal diagnosis. This study was carried out as a part of a large ongoing collaborative hemophilia research project.

In this study, the method for screening the factor VIII gene inversion by reverse transcription-polymerase chain reaction (RT-PCR) was developed and tested. RT-PCR and nested PCR in combination of SSCP were used for screening of small molecular defects. The amplified fragment possibly containing mutation was characterized by DNA sequencing. After identification of the molecular defect in the patient, genetic study in other family members was performed by allele specific amplification (ASA) to identify the carrier status.

CHAPTER II

OBJECTIVES

The objectives of this thesis are:

1. To develop a method for mutation detection in factor VIII gene.
2. To identify mutations in the factor VIII gene by mRNA and DNA analyzes.
3. To develop a method for analysis of mutations and carrier detection in affected families.

CHAPTER III

LITERATURE REVIEW

1. A brief history of hemophilia

Hemophilia is a bleeding disorder resulting from congenital deficiency in either factor VIII or factor IX. In the modern history of hemophilia, the first report of hemophilia-like condition was published by John Otto in 1803 (10) and the X-linked nature of the disease which affects males and is transmitted by females was clearly described. In the 1940s and 1950s, coagulation factor VIII and factor IX were identified and early bioassays performed on plasma could thus distinguish hemophilia A from hemophilia B. Hemophilia B was identified by Aggeler *et al.* (11) and Biggs *et al.* (12) in 1952 after the discovery of complementation of the coagulation defect in mixture of plasma from unrelated patients that indicated the existence of two different types of hemophilias. In the 1970s, factor VIII was isolated from its carrier protein, von Willebrand factor (vWF), which could further distinguish hemophilia A and von Willebrand disease (13).

2. Classification of hemophilia

Hemophilia can be broadly divided into 2 classes, hemophilia A and hemophilia B. Hemophilia A accounts for 80 to 85% of cases of hemophilia, making it the commonest severe hereditary disorder of coagulation. It is an X-linked recessive disorder (14) occurs in all ethnic groups without geographic limitations. Its incidence at birth is approximately 1 per 5,000 males (15).

3. Clinical manifestation of hemophilia A

The clinical hallmarks of hemophilia A are joint and muscle hemorrhages, easy bruising and prolonged and potentially fatal hemorrhage after trauma or surgery. The most characteristic sites of bleeding are joints, particularly the major load-bearing articulations (the knees, elbows, ankles, shoulders, and hips) and large muscle groups. Untreated joint hemorrhage will usually lead to severe limitation of motion and may be extended to permanent disability. Bleeding in other sites can cause serious problems by compressing vital structures, e.g. intracranial or paratracheal bleeding, and can be the causes of mortality (14, 16). The disease can be classified into three levels of severity: severe, moderate, mild, correlating with the residual level of coagulation factor VIII activity in the patient's plasma. Normal plasma factor VIII activity usually ranges between 0.5 U/ml and 1.5 U/ml (50-150%), which 1 U/ml corresponds to 100% of the activity found in 1 ml of normal plasma. The major clinical manifestations of hemophilia A and their correlation with factor VIII activity are outlined in Table 1 (10).

4. Diagnosis of hemophilia A

The diagnosis of hemophilia A should be made whenever unusual bleeding is encountered in a male patient. The screening laboratory tests include platelet count, bleeding time, prothrombin time (PT), activated partial thromboplastin time (APTT). Hemophilia A and B patients will show the normal PT, platelet count, and bleeding time but a prolonged APTT. The differentiation of hemophilia A and von Willebrand's disease can usually be made by measuring either von Willebrand factor antigen or ristocetin cofactor assay (17). Both von Willebrand factor antigen and ristocetin cofactor activity are reduced in patients with von Willebrand's disease and

Table 1. Laboratory and clinical manifestations of hemophilia A.

	Severe	Moderate	Mild
Factor VIII activity level* (U/ml)	<0.01	0.01-0.05	>0.05
% incident	70%	15%	15%
Age of onset	≤ 1 year	1-2 years	2 years-adult
Neonatal symptoms	PCB: usually ICH: occasionally	PCB: usually ICH: uncommonly	None Rare
Muscle / joint hemorrhage	Spontaneous, requires no trauma	Requires minor Trauma	Requires major Trauma
CNS hemorrhage	High risk (2-8%)	Moderate risk	Rare
Post-surgical hemorrhage (without prophylaxis)	Frank bleeding, Severe	Wound bleeding, Common	Wound bleeding with factor VIII activity < 0.3U/mL
Oral hemorrhage following trauma, tooth extraction	Usual	Common	Often

* normal level = 0.5-1.5 U/ml

PCB, post-circumcisional bleeding; ICH, intracranial hemorrhage; CNS, central nervous system.

are normal or increased in patient with hemophilia A. The factor VIII and factor IX coagulant assay are needed to differentiate hemophilia A from hemophilia B.

5. The role of factor VIII in blood coagulation

Blood coagulation is needed to form blood clots to prevent bleeding after damage of blood vessels. This mechanism is based on the 'Cascade' or 'Waterfall' (18) hypothesis, in which blood coagulation proceeds through a series of sequential enzymatic reactions (Figure 1). The cascade can be considered in three parts, the intrinsic and extrinsic systems, and the final common pathway of thrombin formation. The intrinsic system is initiated when blood comes into contact with non-endothelial surfaces and factor XI was activated in the presence of factor XII, pre-kallikrein and high-molecular weight kininogen (HMWK). Activated factor XI, a serine protease enzyme, would convert factor IX into activated factor IX. After released from vWF, factor VIII becomes part of the 'tenase' complex (the calcium-dependent complex of factor VIIIa, factor IXa, and phospholipid), the first molecule in the final common pathway. The role of factor VIII in coagulation is to accelerate the rate of cleavage of factor X by activated factor IX. The extrinsic system provides a rapid response to injury in which activated factor X occurs almost immediately. Factor X is activated by a complex containing tissue factor, activated factor VII, calcium and phospholipid. The intrinsic and extrinsic systems converge on the activation of factor X. Activated factor X forms a complex with factor V, calcium, and phospholipid (prothrombinase complex) to generate thrombin from prothrombin. Thrombin is essential for the conversion of fibrinogen to fibrin. The fibrin clot is stabilized by cross-linking which is catalyzed by the reaction of activated factor XIII.

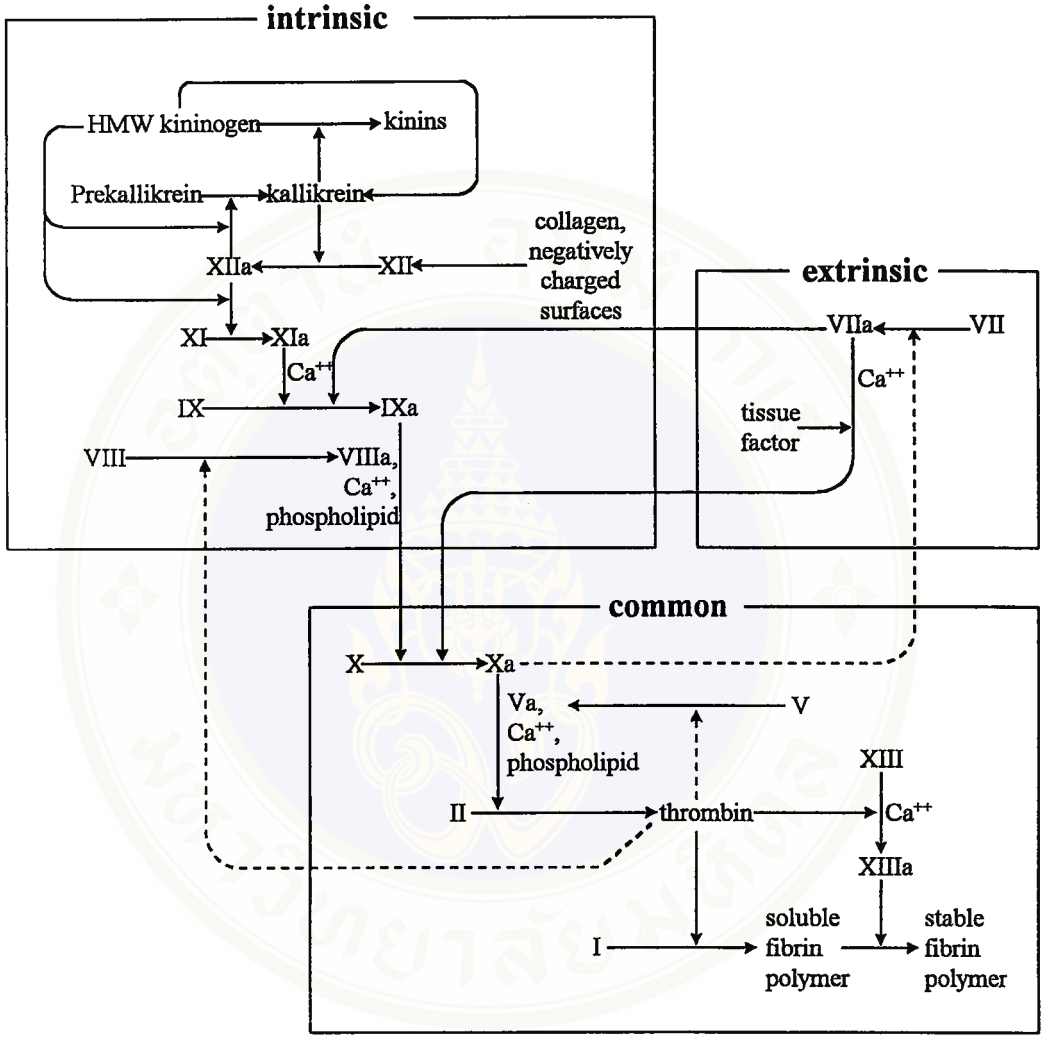


Figure 1. The coagulation cascade (19).

Simplified diagram of the coagulation cascade. 'a' denotes the active form of the coagulation factor; -----> indicates positive feed back by factor Xa and thrombin.

6. The factor VIII gene and protein

6.1 The factor VIII gene

The human factor VIII gene was cloned in 1984 by two independent groups (5, 20). It was located to the most distal end of the long arm of the X chromosome, Xq28 and mapped distal to G6PD and far from the Xq telomere approximately 1 Mb (3, 4). As shown in Figure 2, the factor VIII gene spans 186 kb, comprises 26 exons and as such is one of the largest genes currently known (9). The exon length varies from 69 to 262 nucleotides, except for exons 14 and 26 containing 3,106 and 1,958 nucleotides, respectively. The exons are separated by introns (or IVSs) that also vary considerably in size from 200 bp (intron 17) to 32.4 kb (intron 22). The large IVS 22 is unusual because it contains a CpG island that is associated with two additional transcripts, termed F8A (21) and F8B (22). F8A has a transcript of 1.8 kb and produced abundantly in a wide variety of tissues. The orientation of F8A is opposite to that of the factor VIII gene and its genomic DNA contains no introns. F8B has a transcript of 2.5 kb and is transcribed in the same direction as factor VIII gene, using a private exon plus exons 23-26 of the factor VIII gene. Furthermore, two additional copies of F8A have been found in the human genome and they are located approximately 400 kb telomeric to the factor VIII gene (4, 21). The function of the F8A and F8B transcripts and of their potential protein products is unknown. Factor VIII mRNA is approximately 9 kb in length, which encodes a polypeptide chain of 2,351 amino acids (5, 20, 23). This includes a signal peptide of 19 and a mature protein of 2,332 amino acids, of which the calculated molecular weight (without carbohydrate) is 265 kDa.

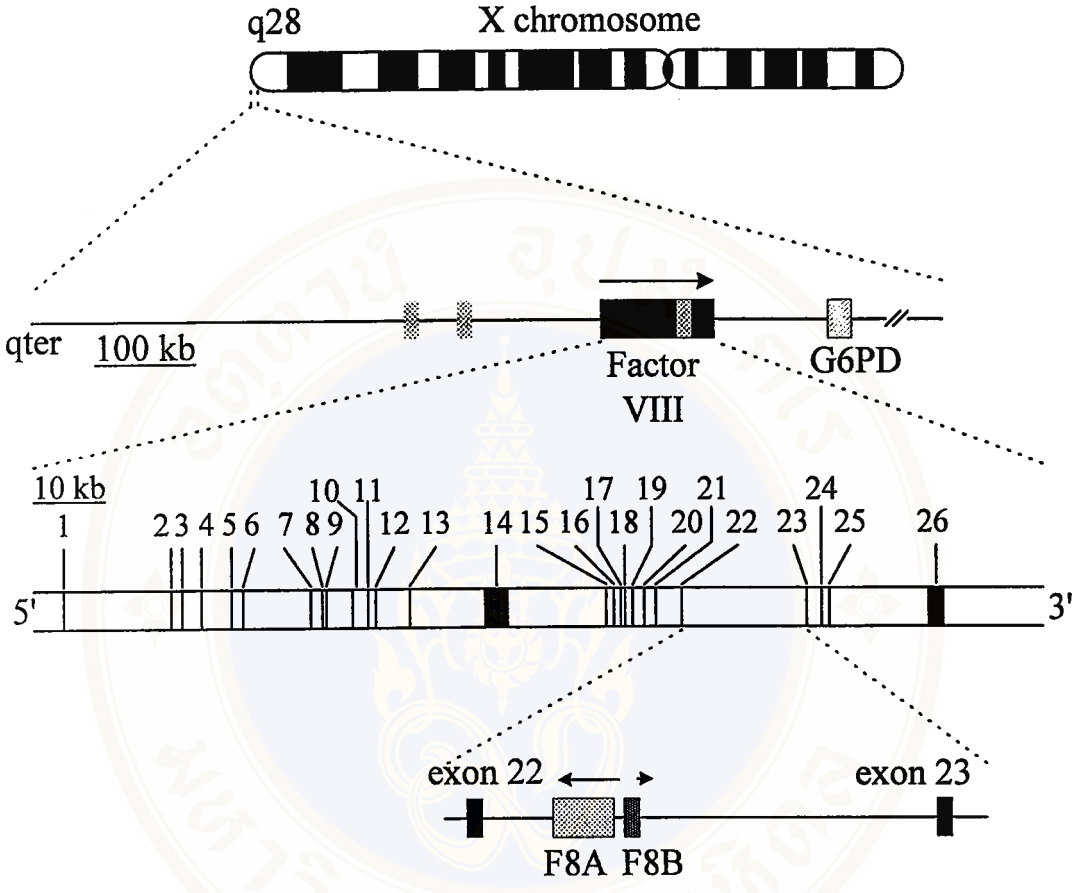


Figure 2. The structure of factor VIII gene (9).

Schematic representation of chromosomal localization and structure of factor VIII gene. The gene is located about 1,000 kb from the Xqter. It is 186 kb long and contains 26 exons. In the large intron 22, there are two nested genes, the intronless F8A and F8B. The direction of transcription is indicated by arrow.

6.2 Structure of factor VIII

Factor VIII is a large multidomain protein containing internal repeats (Figure 3) (6). There are three homologous A-type domains (A1, A2, and A3), defined approximately by residue positions 1-336, 375-719, and 1691-2025, respectively (24). The A domains display approximately 30% homology to each other. These domains further display a similar extent of homology to the copper-binding protein ceruloplasmin and to factor V, the cofactor in the prothrombinase complex (25). The A domains are bordered by short spacers (*a*1, *a*2, and *a*3) that contain clusters of Asp and Glu residues, the so-called acidic regions. The acidic peptide *a*1 spans 337-374 and separates A1 from A2. The second acidic peptide, *a*2 (720-740), connects A2 with the large, heavily-glycosylated B domain, which encompasses approximately residues 741-1648. The third short acidic peptide, *a*3 (1649-1690), connects the B domain with the A3 domain. The B domain is unique in that it exhibits no significant homology with any other known protein. Finally, there are two homologous C-terminal domains (C1 and C2) each of approximately 155 amino acids. The C domains are structurally related to the C domains of the factor V. In addition, the lipid-binding lectin discoidin I, human and murine milk fat globule proteins, and a putative neuronal cell adhesion molecule from *Xenopus laevis* share amino acid sequence similarity to the factor VIII C domains (26-28). The domain organization of the factor VIII polypeptide can thus be designated as A1.*a*1.A2.*a*2.B.*a*3.A3.C1.C2 (6, 20, 23). The locations of seven disulphide bonds within the factor VIII molecule have been reported; there are two in each of the A1, A2, and A3 domains and one within the C1 domain (Figure 3).

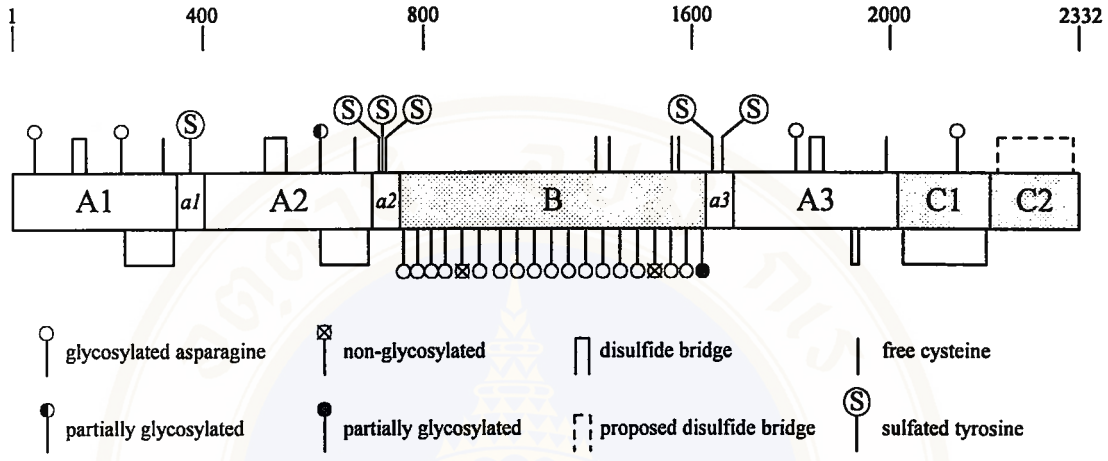


Figure 3. The factor VIII protein (29).

Mature factor VIII consists of 2332 amino acids, which are arranged in a discrete domain structure, A1, A2, B, A3, C1, and C2. The A domains are bordered by acidic regions, *a*1, *a*2, and *a*3. Using B-domainless factor VIII, seven disulfide bonds have been identified: residues 153 and 179, 248 and 329 (A1 domain), 528 and 554, 630 and 711 (A2 domain), 1832 and 1858, 1899 and 1903 (A3 domain), and 2021 and 2169 (C1 domain). Within the C2 domain, residues 2174 and 2326 most likely also form a disulfide bridge. The factor VIII contains 25 consensus sequences (Asn-X-Thr/Ser) that allow N-linked glycosylation. The acidic regions contain consensus sequences that allow sulfation of Tyr-residues at positions 346 (*a*1 region), 718, 719, 723 (*a*2 region), 1664, and 1680 (*a*3 region).

Various interaction sites have been defined on the factor VIII (24, 29). Factor IXa binding is attributed to the A2 domain sequence 558 to 565 and a region within its carboxyterminal part (residues 698 to 710). Besides factor VIII heavy chain, the light chain also contributes to factor IXa binding. By using synthetic peptides, the A3 domain sequence 1811 to 1818 has been identified as a site that binds factor IXa. Collectively, by combining biochemical data with the three-dimensional models of factor IXa and factor VIII, it appears that the factor VIII A2 domain binds to the factor IXa heavy chain (serine protease domain), and the factor VIII A3 domain to the factor IXa light chain (EGF1 domain). In addition, an activated protein C (APC) binding site has been described in the A3 within H2007-V2016. Two peptide regions of factor VIII are implicated to be involved in binding vWF: one at the amino terminal end of intact factor VIII light chain (residues 1649-1671), and the other at the carboxy terminal end (residues 2303-2332). Both ends of factor VIII light chain act synergistically in the binding of vWF. Phospholipid binds to the factor VIII C2 domain in the region 2303-2332. A recent report also identified a region of the C-terminal portion of A1 (T291-F309), which may be involved in binding to a chaperonin, Ig-binding protein (BiP); this protein is associated with inhibition of factor VIII secretion.

6.3 Biosynthesis of factor VIII

Several tissues have the potential of expressing the factor VIII gene. Factor VIII mRNA has been demonstrated in a variety of tissues including spleen, lymph nodes, liver, and kidney (22, 30, 31). However, several lines of evidence indicate that, within the liver, hepatocytes are the major factor VIII-producing cells, whereas organs such as lung and spleen indeed contribute to the presence of circulating factor VIII (30, 32).

6.4 Secretion of factor VIII

The factor VIII is generally poorly expressed due to a low level of steady-state mRNA, and is inefficiently secreted. The initial stage of secretion involves the translocation of the mature 2332 amino acid polypeptide into the lumen of the endoplasmic reticulum (ER), where *N*-linked glycosylation occurs. The transport of the factor VIII from the ER to the Golgi apparatus is limited because the factor VIII appears to interact with a number of chaperone proteins, including calreticulin, calnexin, and BiP. Due to the interaction with these chaperone proteins, a significant proportion of the factor VIII molecules is retained within the ER. Within the Golgi apparatus, factor VIII is subjected to further processing, including modification of the *N*-linked oligosaccharides to complex-type structure, *O*-linked glycosylation, and sulfation of specific Tyr-residues (Figure 3). In addition, the middle part and the carboxy terminal region of the B domain will undergo intracellular proteolysis. These regions comprise a motif (Arg-X-X-Arg), which allows proteolysis by undefined endoprotease at Arg 1313 and at Arg 1648. The latter event disrupts the covalent linkage of the factor VIII heavy chain (A1-*a1*-A2-*a2*-B) and light chain (*a3*-A3-C1-C2), giving rise to the heterodimeric molecule that circulates in plasma. In plasma, the factor VIII circulates as a series of N-terminal heavy chain/C-terminal light chain heterodimers that are dependent upon a divalent cationic association.

6.5 Function of factor VIII

Immediately after its release into the circulation, the factor VIII heterodimer interacts with its carrier protein, vWF, to form a tight, noncovalent complex (Figure 4). The function of factor VIII is to accelerate the rate of cleavage of factor X by activated factor IX (FIXa) on a suitable phospholipid surface, thus amplifying many

folds of the clotting stimulus (2, 33). Prior to secretion, the factor VIII is cleaved at the B/*a*3 boundary (Arg1648-Asp1649) so that plasma factor VIII is a heterodimer with a heavy chain consisting of A1-*a*1-A2-*a*2-B and a light chain consisting of *a*3-A3-C1-C2. The heavy chain, however, is of variable length since the B domain is also cleaved at variable internal positions. In conversion of the factor VIII to its active form to participate in coagulation, plasma factor VIII must first be proteolytically cleaved by thrombin or factor Xa at the *a*1/A2 (Arg372-Ser373) and the *a*3/A3 (Arg1689-Ser1690) boundaries (Figure 5). The first cleavage transforms factor VIII into a three chain molecule and allows the short acidic linker *a*1 to function as a binding-site for A2 in the heterotrimeric active factor VIIIa species (34), while the second releases the factor VIII from vWF by removing the acidic linker *a*3 bearing a vWF binding-site (35). Activation is also accompanied by cleavage at the *a*2/B boundary (Arg740-Ser741), so that activated factor VIII is a heterotrimer formed by A1-*a*1, A2-*a*2 and A3-C1-C2. The first chain is bound to the third by salt bridges, while the A2-*a*2 chain is held in the complex by interaction with *a*1 acidic peptide of the A1-*a*1 chain. The activated factor VIII (VIIIa) is an unstable molecule that rapidly loses cofactor function. The instability of the factor VIIIa results from A2 subunit dissociated from the factor VIIIa heterotrimer when the protein is held at a physiologic pH (34). The factor VIIIa is also inactivated by proteolytic cleavages by APC, an important regulator of coagulation, at Arg336 and Arg562 (36).

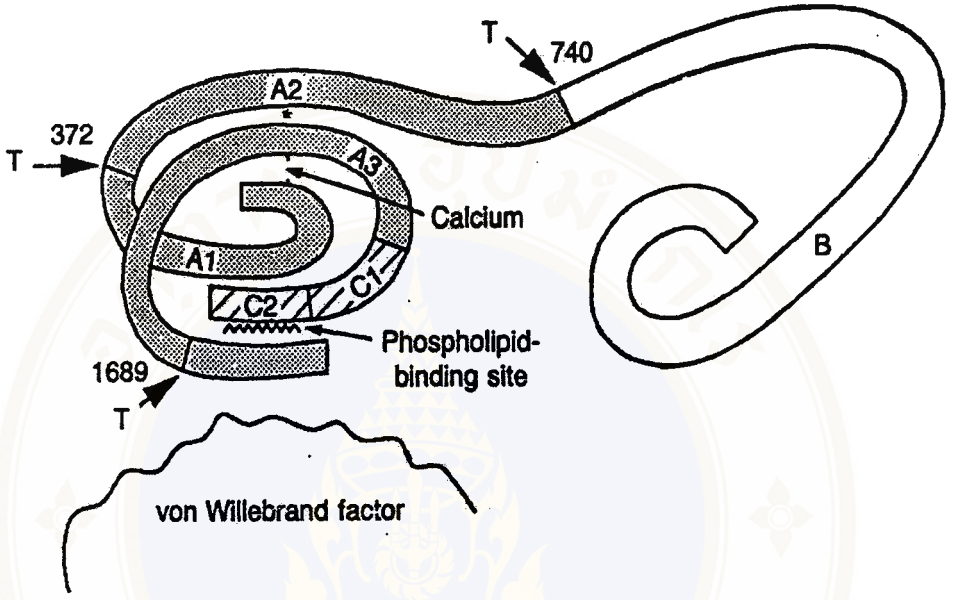


Figure 4. Model of the factor VIII and von Willebrand factor (vWF) interaction (14).

The figure shows the interaction between the factor VIII and vWF. After cleavage by thrombin (indicated by arrows and T), the activated factor VIII is released from vWF.

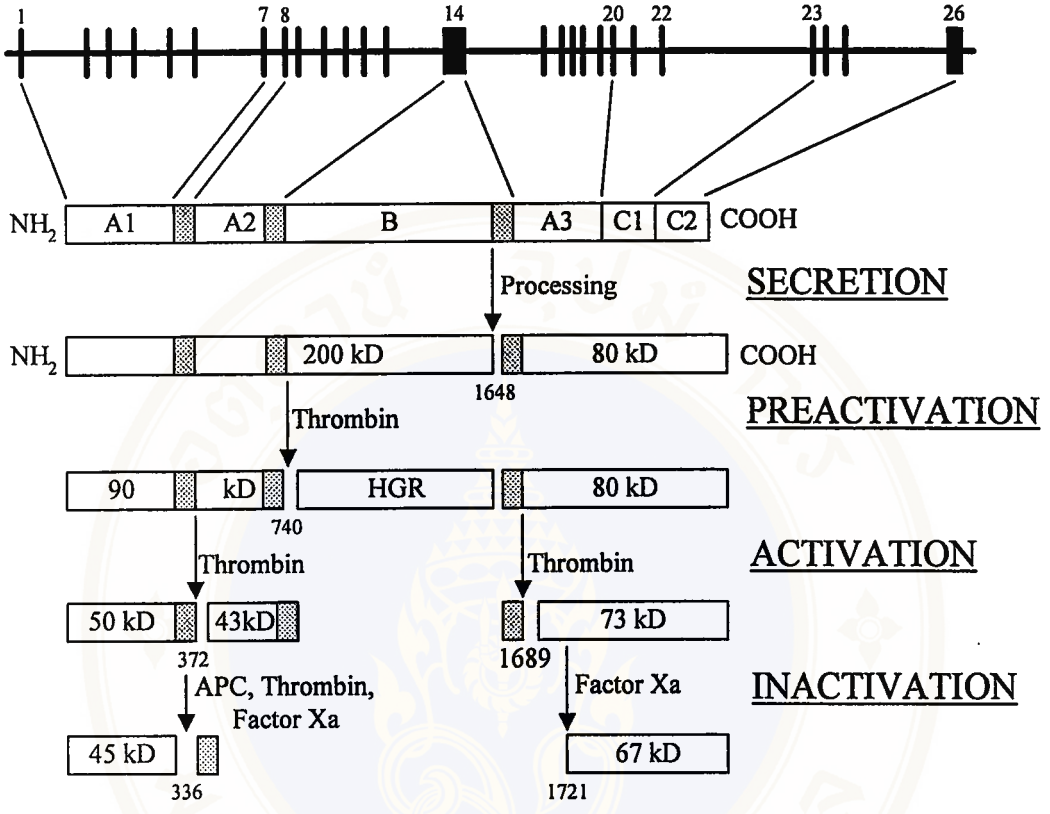


Figure 5. The fate of factor VIII protein (37).

The top line depicts the gene region and vertical lines mark the 26 exons. The factor VIII protein domains are encoded from exons indicated by the connecting lines. The heavy and light chains of the gene product are cleaved by proteases, and the double chain protein secreted into plasma is activated by thrombin. The mature protein is whittled down and inactivated through successive cleavages by thrombin, activated protein C (APC), and factor Xa. HGR stands for the expandable heavily glycosylated region encoded by exon 14.

7. The molecular pathology of hemophilia A

Hemophilia A is a mutationally heterogeneous disease. Characterization of mutations may help to identify amino acids or regions with essential functional or structural properties and thereby clarify the mechanism of pathogenesis. The DNA of more than 1000 hemophilia A patients have been examined for molecular defects and can be broadly divided into gross gene rearrangement, single base substitutions, deletions, and insertions. The mutation database is now available online at the World Wide Web site; http://europium.csc.mrc.ac.uk (8). Table 2 summarizes different types of mutations of the factor VIII gene deposited in the database.

Table 2. Summary of different mutations in factor VIII gene (until June 13, 2000).

Types of mutation	Number of different mutations
Single base substitutions	
Missense	287
Nonsense	56
Splicing defects	35
Deletions	
Small deletions	77
Large deletions	>80
Insertions	28
Total	>563

7.1 Gene rearrangements

7.1 Gene rearrangements

Gene inversion is the main defect of mutations caused by of gross gene rearrangements which responsible for about 25% of all patients with hemophilia A and more than 40% of those with severe disease. The mechanism responsible for this mutation likely involves an intrachromosomal recombination between homologous sequences within intron 22 of the gene and at the sites approximately 400 kb upstream (Figure 6). The sequence involved in this recombination event within intron 22 is the intronless factor VIII-associated gene A (21) that is transcribed in the opposite direction to the factor VIII gene. There are two further copies of gene A located about 400 kb 5' and telomeric to the factor VIII gene (7). A single crossing over event causes a DNA inversion of roughly 600 kb, resulting in the factor VIII gene being divided into two parts, exon 1-22 and exon 23-26, which are widely separated and have opposite directions. Depending upon which extragenic copy of gene A is involved in the crossing over event, two main types of inversions are recognized. Crossover between the most distal sequence A and its IVS 22 homolog results in a type 1 inversion, the most common inversion event (7, 38), which responsible for 35% of severe hemophilia A cases (39). Crossover between the proximal extragenic sequence A and its IVS 22 homolog results in a type 2 inversion, which accounts for a further 7% of the severe cases. Both of these inversions can be detected by Southern blot analysis (7). In addition, a rare Southern blot pattern, about 1%, is thought to result from an individual carrying more than two extragenic copies of gene A.

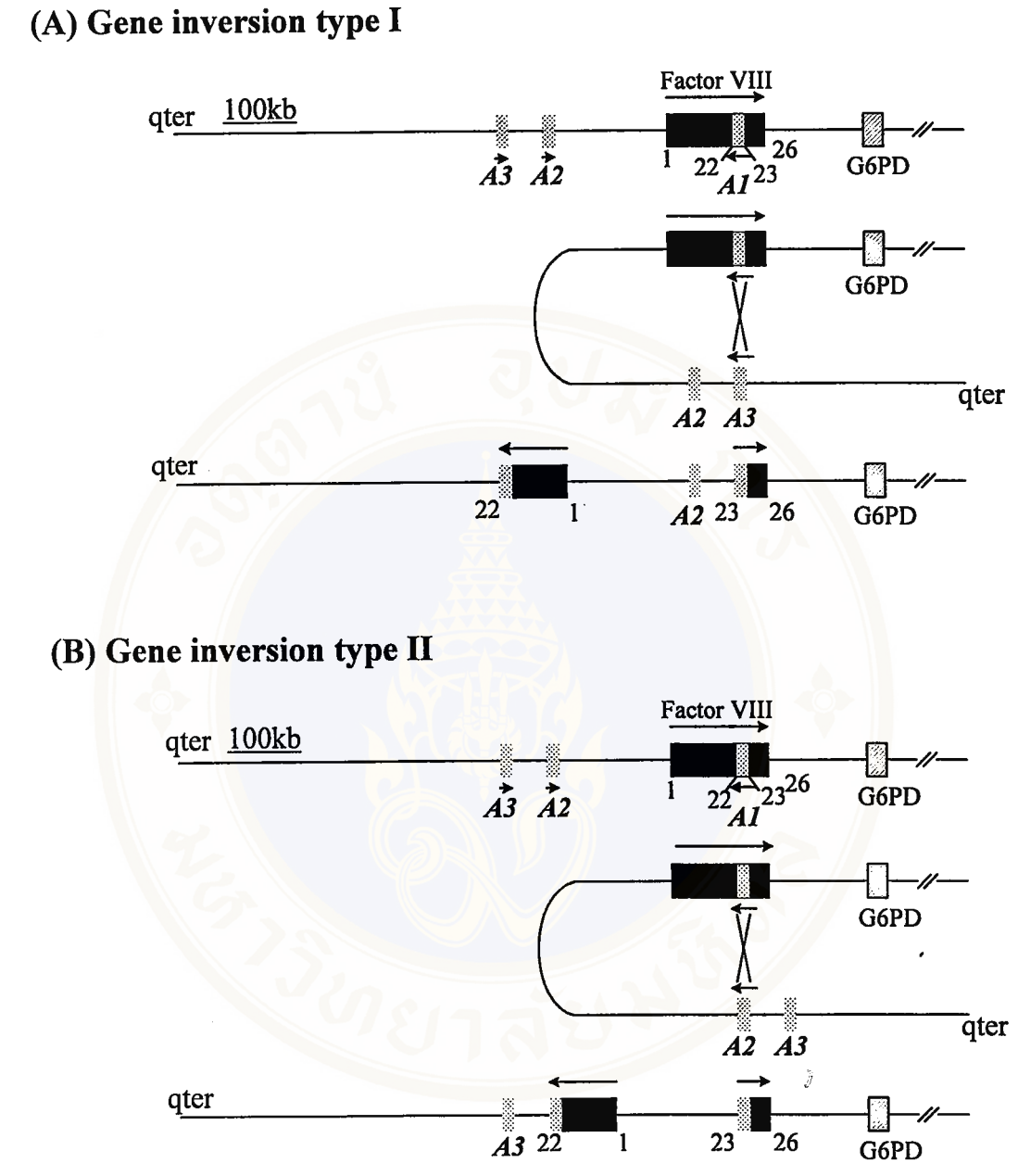


Figure 6. The mechanism of intrachromosomal recombinations resulting in the two common types of the factor VIII gene inversions (40).

Schematic representation of the mechanism of the factor VIII gene inversions caused by intrachromosomal recombination between homologous sequences within intron 22 (*A*1) and at approximately 400 kb telomeric to the factor VIII gene (*A*2 or *A*3). The gene inversion type I occurs from the recombination between *A*1 and *A*3 while the gene inversion type II from *A*1 and *A*2.

7.2 Single base substitutions

At present, there have been 378 different single base substitutions reported in the hemophilia A database. This group of mutations consists of 287 missense mutations, 56 nonsense mutations, and 35 mRNA splicing errors. These mutations are spread throughout the gene. The analysis of missense mutations should gradually help to define the structural features that are important to the transport, processing, stability, and function of factor VIII (41). Nonsense mutations are expected to cause premature termination of translation but recently mRNA analysis has shown that nonsense mutations may sometimes alter RNA processing and cause skipping of the exon containing the nonsense codon (42). The mechanism, significance, and frequency of exon skipping due to nonsense mutations are not known. The mutations may occur at the invariable GT (donor) or AG (acceptor) splice sites. Some of the mutations may activate the cryptic splice site (39). The study of point mutations in the factor VIII gene revealed hotspot at CpG dinucleotides. About 46% of these point mutations have a common substitution CG to TG if the mutation occurs in the sense strand or CG to CA if the mutation occurs in the antisense strand (43). The mutations may occur because, in mammalian DNA, most CpG dinucleotides are methylated (at C-5 of cytosine) by methyltransferase; the subsequent spontaneous deamination of the 5-methylcytosine produces a TpG dinucleotide. It has been estimated that in the factor VIII gene CG to TG or CA mutations are 10-20 times more frequent than mutations of CG to any other dinucleotides (44).

7.3 Sequence deletions

Factor VIII gene deletions have been divided into large (more than 100 nucleotides) deletions and small (less than 100 nucleotides) deletions.

7.3.1 Large deletions

About 5% of patients with hemophilia A have gross deletions in the factor VIII gene with reports of over 80 unique large deletions in the mutation database. These may involve the whole or a part of the gene and the mechanism in most of these is probably non-homologous recombinations (45). In the majority of these defects the deletion breakpoints have not been precisely characterized. Southern blot analysis indicated that there are not many patients with the same breakpoints, suggesting that the mutations do not show obvious preferences for any specific region. Deletions almost always result in severe disease with no factor VIII activity measurable in plasma samples and no antigen detected. However, a deletion of exon 22 (46) and another deletion of exons 23-24 (47, 48) were found to be associated with moderate disease. The mRNAs in these cases are expected to maintain the normal reading frame with subsequent secretion of hypoactive factor VIII lacking 52 or 98 amino acids, respectively.

7.3.2 Small deletions

A total of 77 small deletions have been included in the database. These mutations are distributed fairly steadily through the exons, which about one-third of the mutations are in the large exon 14. The deletions are varying in size from 1 bp to 86 bp. The patients with small deletions are almost all associated with severe disease. Most of these small deletions produce frameshifts that also usually cause premature termination of translation and consequent abolition of factor VIII expression. The majority of the small deletions occur in DNA regions of short direct repeats.

7.4 Sequence insertions

There are 28 different insertions varying in size from 1 bp up to 2.1 kb and 3.8 kb of LINE elements reported in the hemophilia A database. LINE elements, retrotransposon sequences, are composed of about 5% of the human genome and found distributed throughout the genome in approximately 10^5 copies. The full length of the element is 6.1 kb and most of the copies are partial and defective. Insertion of LINE retrotransposons in the factor VIII gene was first reported in two cases of severe hemophilia A (49). In one case a 3.8 kb portion of a LINE element was inserted in exon 14, in another case a 2.1 kb portion of a LINE element was inserted in a different site of the exon 14 with all the characteristics of retrotransposition. The small insertions are often involved an A inserted in a stretch of A residues (50, 51). In exon 2, a 10-bp sequence was found to be a tandem duplication of existing sequence (52). Most of the small insertions occur in DNA regions of short direct repeats. All insertions are associated with severe disease, which may be caused by either gross insertions or predicted frameshifts.

7.5 Carrier detection by DNA-based analysis

The genetic approach for carrier detection and prenatal diagnosis of hemophilia A requires the ability to recognize the abnormal factor VIII gene. Because of the great variability in the hemophilia A gene mutation, carrier detection was largely performed using intragenic and extragenic linkage analysis of DNA polymorphisms. Restriction fragment length polymorphisms (RFLPs) were the first type of genetic marker used to track hemophilia inheritance (53). First analyzed by Southern blotting, these polymorphisms are now much more commonly analyzed by restriction enzyme digestion of amplified DNA. The most commonly used RFLPs are *Bcl* I in intron 18,

Hind III in intron 19, *Xba* I in intron 22, and *Bgl* I in intron 25 (54). In addition to detection of RFLPs, allele specific oligonucleotide hybridization (ASO) can be used to detect a G/A polymorphic site in intron 7 of the factor VIII gene (55). Not only intragenic polymorphism detection, but the extragenic markers, e.g. DX13 (DXS15) or St14 (DXS52) were also used. Although initially useful in gene tracking, it was found that there is a significant recombination within the factor VIII gene, leading to the risk of misdiagnosis. Another kind of gene marker, dinucleotide tandem repeats with multiple alleles detectable as length polymorphisms, is also used in the linkage analysis of hemophilia A. These repeat regions in introns 13 and 22 are most useful for tracking factor VIII gene inheritance (56, 57). These polymorphisms are variably informative since different allele frequencies are found in different ethnic groups and different proportions of females are heterozygous.

Although linkage analysis is generally technically simple and is applicable to the majority of families, there are several limitations to its usefulness. These techniques are useful when an affected male patient and his intervening family members are available for analysis. In some families with limited pedigrees and with only one affected male patient, females can only be excluded from being carriers when they have not inherited the allele with hemophilia A. Lack of informative polymorphisms also results in an inability to diagnose the carrier state. Linkage disequilibrium of several polymorphisms occurring during meiosis also leads to the limitation of this method (54).

7.6 Development of methods for direct mutation analysis

After the cloning of the factor VIII gene in 1984, initial studies identified the mutations that involved either partial or total deletions of the gene or nucleotide

substitutions affecting the recognition sequence of the *Taq* I restriction enzyme (58). This restriction enzyme proved relatively efficient at detecting some point mutations since the *Taq* I recognition sequence, TCGA, contains a CpG dinucleotide that often represents a hotspot for transitions of CG to TG or CA and in particular CGA to TGA (Arg to stop) (43). Later studies using mutation screening methods on polymerase chain reaction (PCR)-amplified factor VIII sequences significantly improved the yield of mutations identified. Using denaturing gradient gel electrophoresis (DGGE) of all exons, most exon/intron boundaries, and the putative promoter, Higuchi *et al.* (50, 59) were able to identify mutations in 86-94% of patients with mild and moderate hemophilia A, but in only 53% of the severely affected patients. However, the size and complexity of the factor VIII gene continued to be an obstacle to a thorough and complete analysis of all the relevant sequences of the gene. To reduce the extent of the factor VIII gene to be screened, Naylor *et al.* (60) used RT-PCR of illegitimate transcripts of the factor VIII gene to obtain eight segments comprising the essential gene sequences. The segments were screened for mutations by chemical mismatch detection. This method was extremely efficient. It was able to detect mutations in all 30 hemophilia A patients and also revealed that about half of severely affected patients had the mRNA with exon 22 not contiguous to exon 23 (61). Lakich *et al.* (7) and Naylor *et al.* (62) found that these patients had an inversion involving the factor VIII gene that could be detected by Southern blot analysis using a probe corresponding to F8A region of intron 22. David *et al.* (63) used single-strand conformation polymorphism (SSCP) to screen mutations in twelve unselected hemophilia A patients and can located the mutations in five patients. Lin *et al.* (52) used a combination of SSCP and dideoxy fingerprinting (ddF) for each exon of the factor VIII gene to

characterize the molecular defects in 85% of hemophilia A patients. Conformation sensitive gel electrophoresis (CSGE) was applied to detect the mutations in a group of seven non-inversion severe hemophilia A patients. Mutations were located in 6/7 patients by CSGE, the seventh had an intragenic deletion (64). Detection of mutations in the patients enabled carrier status to be determined in 20 female relatives. Several different screening methods have been used to identify the mutations in the factor VIII gene. The principles, the relative merits, and drawbacks of each technique are addressed in the following section.

8. Methods for detection of mutations

Two technological developments have revolutionized the mutation analysis. Firstly, cloning and characterization of the disease genes led to direct screening of the mutation in the gene sequence rather than tracking the inheritance of mutant chromosome through families. The second is the introduction of the polymerase chain reaction (PCR), which can amplify a segment of DNA to be more than one million-fold for analysis. The methods of mutation detection can be broadly divided into two categories, methods for detection of known and unknown mutations.

8.1 The methods for detection of known mutations

A variety of methods based on the PCR technique have been developed to detect known mutations and polymorphisms.

8.1.1 Allele-specific amplification (ASA)

Synonymous terms for ASA include, PCR amplification of specific allele (PASA), allele-specific PCR (ASP), and the amplification refractory mutation system (ARMS). The ASA technique is based on the oligonucleotide primers with

mismatched 3' termini which will not function as PCR amplimers under appropriate conditions (65). Paired PCR reactions are carried out. One primer (the common primer) is the same in both reactions, the other (the allele-speccific primer) exists in two slightly different versions, one specific for the normal sequence and the other specific for the mutant sequence. Specific amplification is obtained if the allele-specific primer matches to the desired allele, but mismatches to the other allele at its 3' end (Figure 7). Since *Taq* DNA polymerase lacks a 3'→5' exonuclease activity, the mismatch prevents efficient 3' elongation by this enzyme. Thus, single base change, deletion, and insertion in DNA can be detected rapidly. The location of the common primer can be chosen to give a product of any desired size, so that it is easy to design multiplex reaction systems (66).

8.1.2 Allele-specific oligonucleotide (ASO) hybridization analysis

Dot blots

This technique is performed by hybridization allele-specific oligonucleotide (ASOs) probes to denatured PCR-amplified DNA dotted on a nitrocellulose or nylon membrane (67). The detection of hybridization is via a signal generation from the label linked to the ASO probe, such as a radiolabel, fluorophore or biotin. Autoradiography, fluorescence and conjugation to an avidin- or streptavidin-enzyme conjugate are the methods to detect these labels, respectively. With appropriate ASO probe and hybridization condition, the test is highly accurate. However, this technique has been superseded by other methods because of the need for additional technical manipulations subsequent to the PCR procedure. In addition, a down-stream signal generation system is required and a typical test is restricted to the analysis of a single allelic variation. It is also impractical to establish a generic protocol for ASO

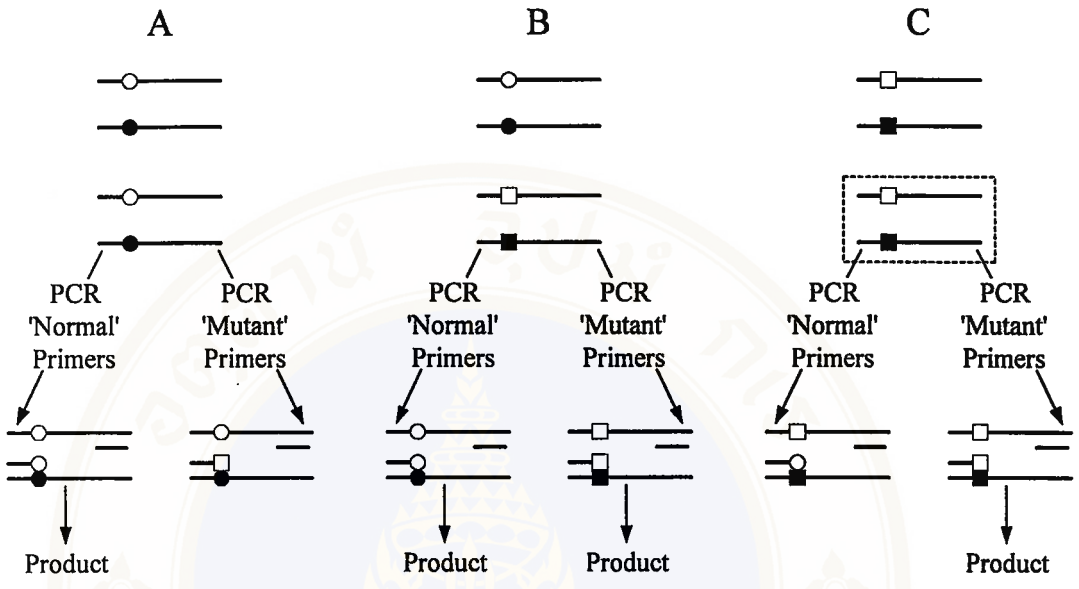


Figure 7. The principle of allele specific amplification (ASA) technique (67).

Schematic representation of the principle of the ASA assay,: (A) is normal homozygote DNA; (B) is heterozygote DNA; (C) is affected homozygote or hemizygote DNA ( ). Circles represent the appropriate nucleotide of the normal allele and squares represent the equivalent nucleotide of the mutant allele. Open and filled circles are complementary base pairs. Similarly, open and filled squares are complementary (i .e. a circle paired with a square is mismatched).

hybridization since the hybridization conditions are always dependent on the melting temperature of the probe/target duplex, which will vary from one mutation or polymorphism to another.

Reverse dot blots

This method is similar to the dot blot hybridized with ASO probe but the format is inverted. Reverse dot blots permit the simultaneous analysis of several mutations or polymorphisms in a single test. Various ASO probes are immobilized onto a membrane and hybridized by biotinylated PCR product. After hybridization, the PCR product that binds to the probe on the membrane is detected by avidin- or streptavidin enzyme conjugate (Figure 8). However, the limitations of this technique are as for the dot blot hybridization (67).

8.1.3 RFLP analysis

This technique is based on the principle that some mutation will create or abolish a restriction enzyme site. Traditionally, restriction fragment length polymorphism (RFLP) analysis was carried out using Southern blotting, but the technique may take up to a week to perform (69). Once an RFLP has been identified, it can now be detected by PCR rather than by Southern blotting, as long as some of the surrounding DNA sequence is known. If the surrounding sequence is not known, it may be determined by cloning and sequencing the restriction fragments detected by the probe. The surrounding sequence, once determined, can then be used for PCR primers design. After PCR, the product is digested with the appropriate restriction enzyme. Electrophoretic separation of digested DNA demonstrates the presence or absence of the restriction site. With this type of analysis, it is useful to have a control for the restriction digestion in order to avoid a misdiagnosis.

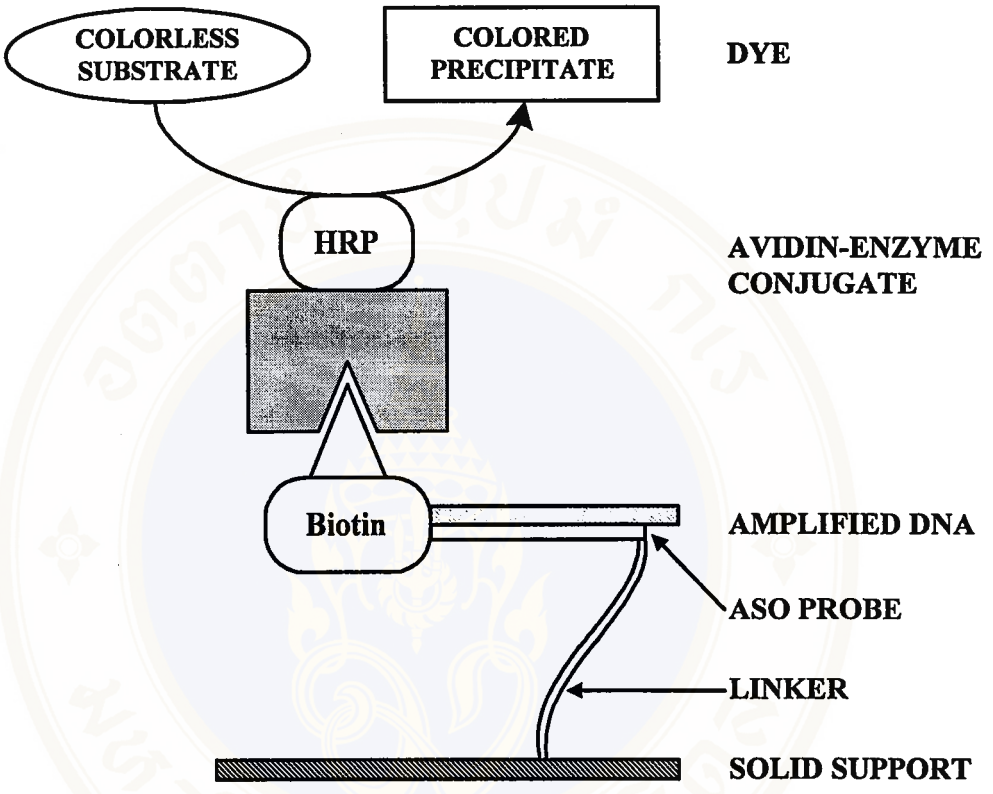


Figure 8. Schematic of reverse dot blot hybridization technique (68).

A biotinylated PCR product is hybridized to an allele-specific oligonucleotide (ASO) probe. The probe is bound to a nylon membrane by poly (dT) and UV cross-linking or by covalent attachment with amino linkers. The hybrids may be detected by reaction of a colorless substrate to form a colored precipitate.

8.1.4 Competitive oligonucleotide priming (COP)

Competitive oligonucleotide priming (67) employs allele-specific primers designed in the same manner as ASO probes. The allele specific amplification is achieved from an appropriately variant nucleotide matched roughly at the middle of each primer of a primer pair (Figure 9). However, the primers comprising the pair must be distinguishable, for example one can be radiolabelled. With this format, PCR is carried out and the products analysed by agarose gel electrophoresis. The expected result is an equivalent band in all samples analysed. To discriminate between alleles, the gel is dried and autoradiographed. A signal appears on the X-ray film that corresponds to the incorporation of the radiolabeled primer into the PCR product. Thus, if the 'normal' primer is [^{32}P]-labeled, the originating genomic DNA can either be from homozygous or heterozygous individuals. The drawback using this method is the difficulty of distinguishing accurately between homozygotes and heterozygotes. To overcome this problem, the individual primers of the primer pair may be conjugated to different and distinguishable signal generation labels such as fluorescein and rhodamine.

8.1.5 Primer extension sequence test (PEST)

This method of mutation detection, like COP, relies on the extension of an allele-specific primer whose allele-specific nucleotide is within the primer. Thus the extension, or non-extension, of the primer relies on the hybridization of the primer to the template (69). An ASO for the 'mutant' allele is used in PCR after precision optimization such that the ASO is extended only on the correctly matched template and generates a product with a downstream PCR primer. A further primer, upstream

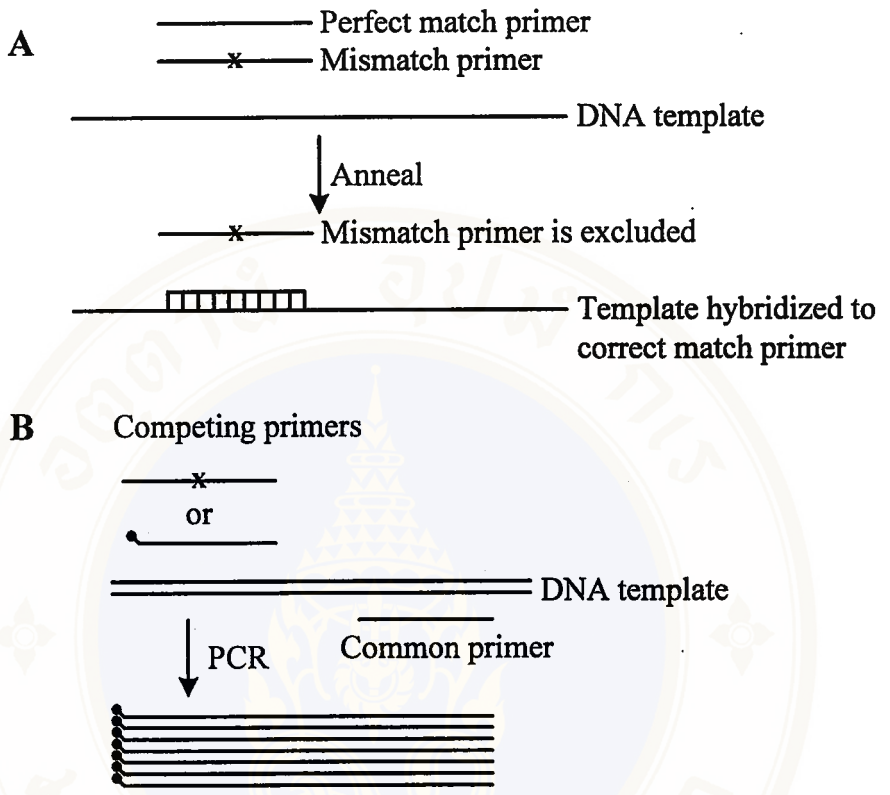


Figure 9. Schematic of competitive oligonucleotide priming (67).

(A) Two oligonucleotide primers differing by one nucleotide (shown as x) are combined with a DNA template. The perfect match primer preferentially anneals to the template excluding the mismatched primer. (B) One of the primer pair, the correctly matched primer, is labeled (•). This labeled primer excludes the mismatched primer and when combined with a common primer in PCR is incorporated into product. Identification of the label incorporated into the product allows inference of the template sequence.

of the polymorphism/mutation, complementary to an invariant sequence and of the same sense as the ASO is also included. If the ASO does not hybridize, a PCR product is generated with the upstream primer. If the ASO does hybridize, it generates the PCR product itself. Heterozygous DNA templates are characterized by incorporation of both primers into PCR products. Since one primer is upstream of the other, products of different sizes are generated and may be resolved easily by electrophoresis (Figure 10). The drawbacks of this system are that only one mutation can be analyzed per reaction and that each reaction must be optimized for the respective mutation, since the T_ms for different ASOs vary. It is also likely that specificity problem will be encountered due to the non-competitive annealing of the ASO to template DNA.

8.1.6 Mutation detection using *Taq* 5'→3' exonuclease activity

Taq DNA polymerase does not possess a 3' to 5' exonuclease activity, but does exhibit a 5' to 3' exonuclease activity. These properties have been exploited to demonstrate the presence of a specific target DNA as the PCR proceeds (67). This is achieved by including another primer downstream of one of the conventional amplification primers. This additional primer is blocked for extension at the 3'-terminus and labeled at the 5'-terminus. Extension from the primer upstream of the additional primer results in detectable liberation of the 5'-label (Figure 11). The disadvantage of this method is the inability to multiplex the reaction. In addition, for this type of application the method would require the allele specificity to reside within the additional, 3'-blocked, 5'-labeled primer. This in turn would require this primer being designed in two forms, as for an ASO probe.

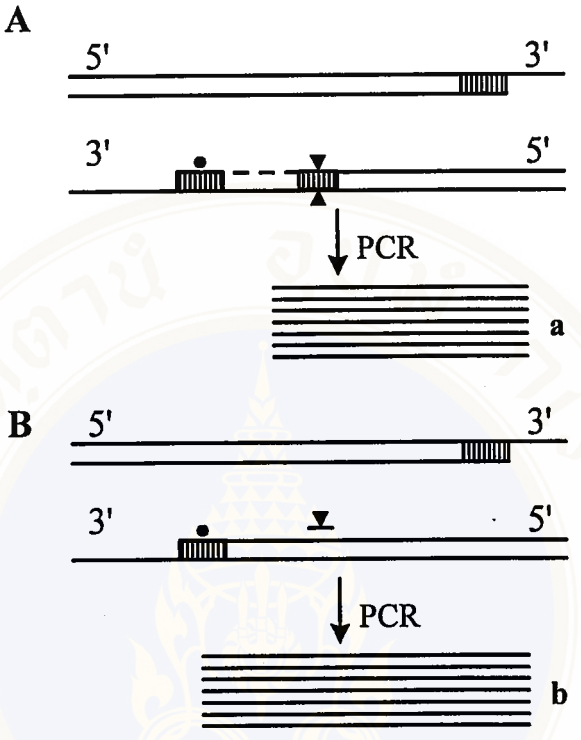


Figure 10. Schematic of primer extension sequence test (67).

Closely-spaced vertical lines indicate the base-pairing of either mutant oligonucleotide (▼) or upstream primer (•). (A) The mutant oligonucleotide is complementary to the template DNA, blocking extension of the upstream primer, and extended to give short PCR product 'a'. (B) The mutant oligonucleotide is not complementary to the template and does not hybridize. A larger PCR product 'b' is generated from the primer upstream of the mutation.

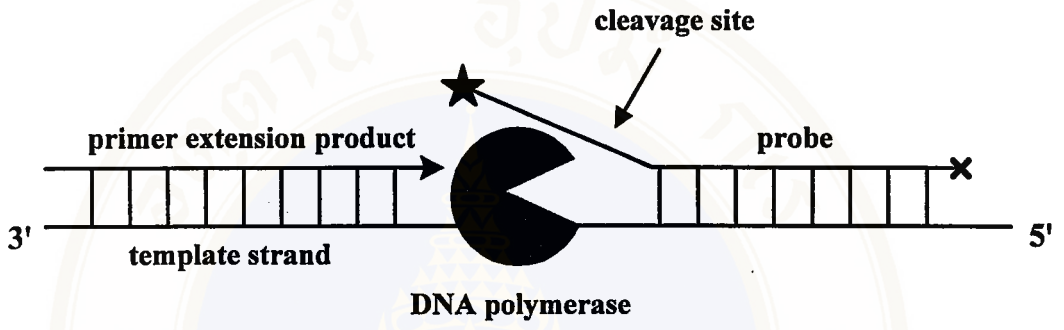


Figure 11. Mutation detection using *Taq* 5'→3' exonuclease activity (67).

Diagram of 5'→3' exonuclease cleavage of 5'-labeled oligonucleotide probe to generate labeled fragments in a PCR. The asterisk represents the 5'-label on the probe and the X represents the 3'-block on the probe.

8.1.7 Mutation detection by introduction of restriction sites

This method of mutation or polymorphism detection operates by incorporating a deliberate mismatch nucleotide close to the 3' terminus of a PCR primer, adjacent to a mutated / polymorphic nucleotide (67). After incorporation of the primer into a PCR product, the variant nucleotide of one allele complements the sequence of the primer to introduce a restriction enzyme recognition site. The corresponding variant nucleotide of the other allele does not complement the primer and the restriction site is not formed. Subsequent digestion of the PCR product with the restriction enzyme identifies which alleles were present in the template DNA (Figure 12). The limitation of the method involves the possibility of creating a restriction site within the primer in the confines of the genomic DNA sequence, since for some mutations there may not be a potential restriction site for incorporation.

8.2 The method for detection of unknown mutations

Many screening methods have been developed in both sensitivity and efficiency to offer a rapid and accurate test.

8.2.1 Single strand conformation polymorphism (SSCP)

The SSCP technique relies on different mobilities of single-stranded DNA on electrophoresis due to different secondary structures (71, 72). Single-stranded DNA has a tendency to fold up and form complex structures stabilized by weak intramolecular bonds, notably base-pairing hydrogen bonds. This secondary structure has a large effect on the rate of migration during electrophoresis under non-denaturing conditions. Even a single base change can radically alter the secondary structure and

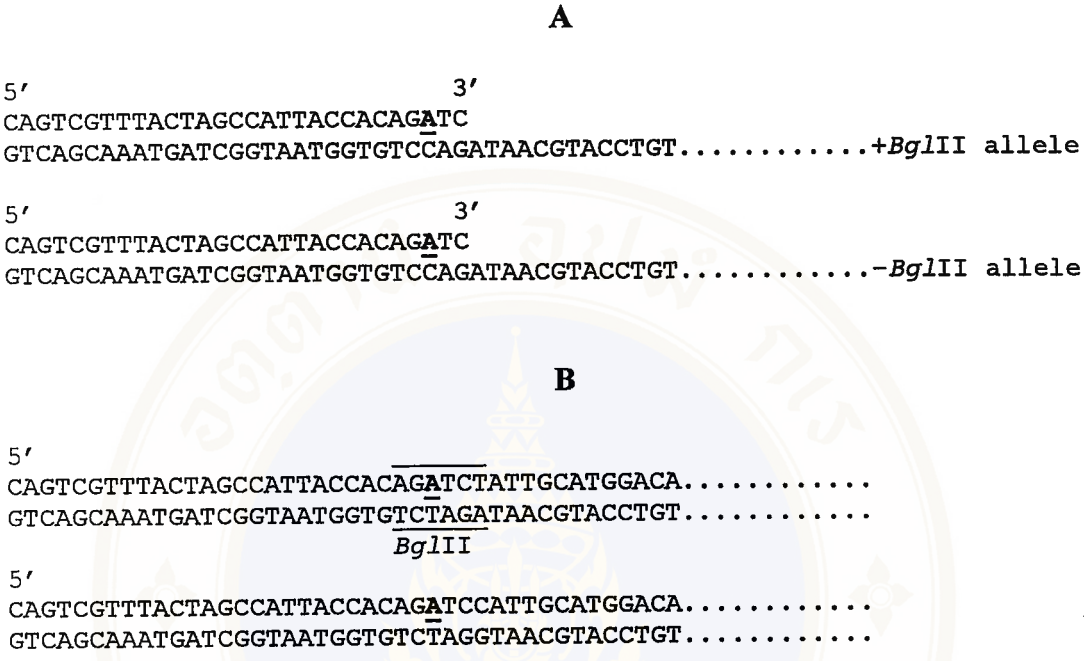


Figure 12. Mutation detection by introduction of restriction sites (67).

(A) PCR primer annealed to + *Bgl*II and –*Bgl*II alleles. The bold and underlined 'A' residue is deliberately changed to generate a partial *Bgl*II restriction recognition site. (B) Extension of the primer and incorporation into PCR product creates a *Bgl*II recognition site (A↓GATCT; under and overlined) from the + *Bgl*II allele but no restriction recognition site is created from priming the – *Bgl*II allele.

hence the rates of migration of a nucleic acid fragment (Figure 13). For SSCP, amplified DNA samples are denatured and loaded on a non-denaturing polyacrylamide gel. Gel composition may be varied by inclusion of glycerol and electrophoresis can be performed at ambient temperature or at 4°C. For efficient detection of sequence alterations, PCR products analyzed must be kept short, preferably less than 300 bp. This can be achieved by use of many overlapping primer pairs that can be radiolabeled or unlabeled, or more practically by sequential restriction enzyme digestion of a large PCR product. A multiplex PCR can be performed to reduce the time necessary for screening the whole gene (51). Detection of the product can be done by radiolabel, ethidium bromide or silver staining (54). This technique has the advantage of simplicity and is widely used for detection of mutation and polymorphism in many genes. Its disadvantages are the requirement to perform analysis under more than one electrophoresis condition to detect all possible conformational changes and its lack of sensitivity at detecting these changes in large PCR products. The sensitivity of this system is variable from region to region, which is typically 60-95% for fragments shorter than 200 bp. This sensitivity can be increased by restriction enzyme digestion by using different enzymes such as restriction endonuclease fingerprinting (REF) (73) or by adding a dideoxy component such as a dideoxy sequencing reaction (74); however, increasing sensitivity through these methods also makes the technique more complex.

8.2.2 Denaturing gradient gel electrophoresis (DGGE)

This technique, described by Fischer and Lerman (76), relies on the property of DNA to denature in domains, and not all at once. This results in a branched structure. In DGGE, DNA is electrophoresed on a polyacrylamide gel with a gradient of

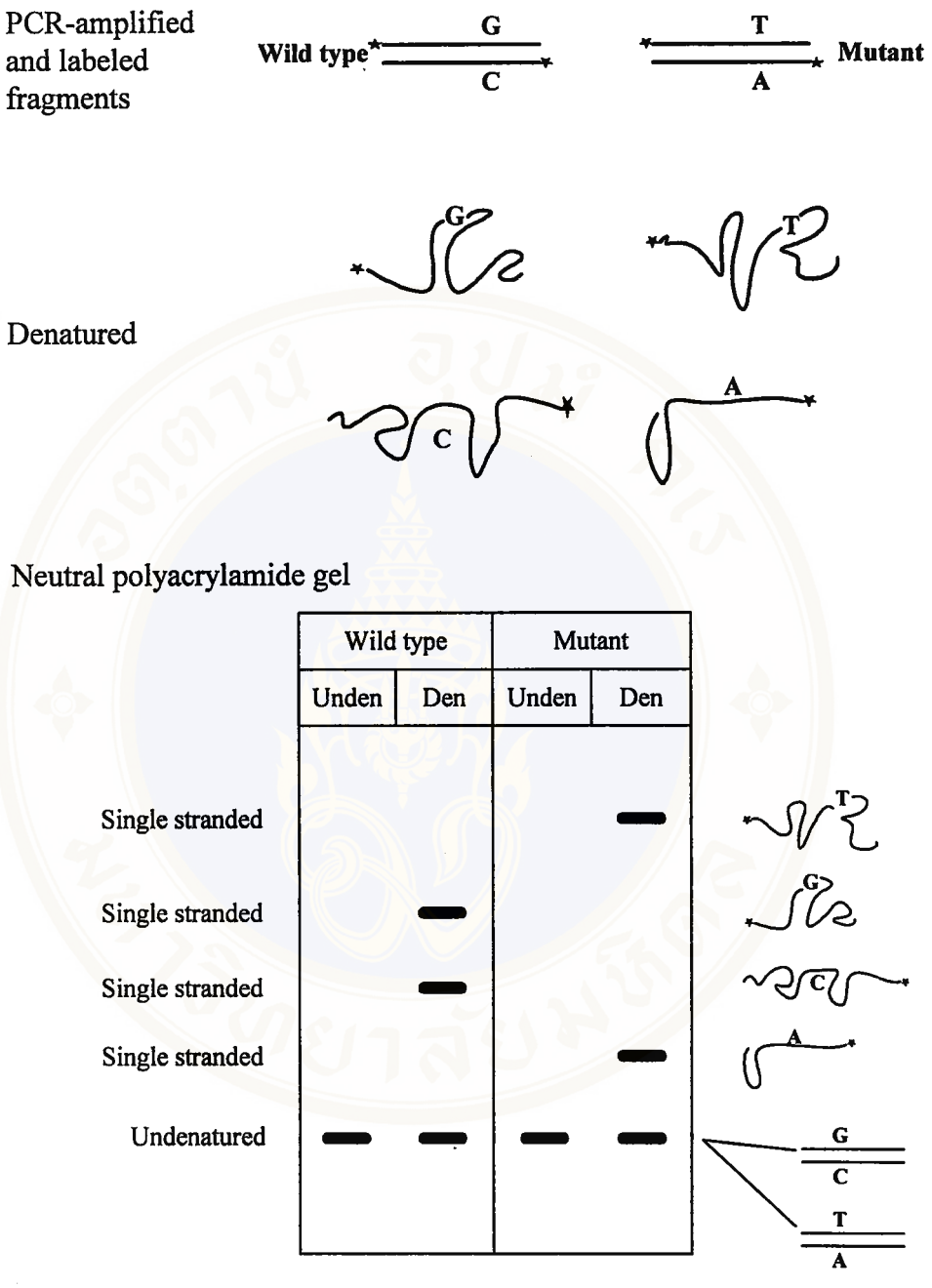


Figure 13. Principle of single strand conformation polymorphism (75).

Labeled double stranded DNAs of wild type and mutant alleles are denatured into single strands and separated on non-denaturing polyacrylamide gel. Mobilities of single stranded DNAs depend on their conformations, which are different between that of wild type and mutant alleles.

denaturants based on increasing temperature or concentration of chemical denaturants such as urea and formamide. Melting of the DNA occurs in groups of 50-300 bp, the so-called "melting domains", that the stability of each melting domain depends on its sequence. DNA duplexes differing even by a single base pair have a different temperature or denaturant concentration at which they melt. When melted, they stop migration in the gel. Therefore, mobilities of the duplexes stop at different positions (corresponding to different concentrations of denaturant/temperature), signaling the mutation (Figure 14). DGGE is potentially highly sensitive, but it requires very careful design of primers, so that the sequence amplified has the right profile of melting domains. Sensitivity is improved by adding a GC-rich fragment, designated a GC-clamp, to one end of the PCR primers (77). The more recent modifications have included utilization of capillary electrophoresis and two-dimensional electrophoresis (78). The use of capillary electrophoresis allows more precision and sensitivity. In the case of the two-dimensional electrophoresis, separation in the first dimension is according to size, while the second dimension is according to DGGE mode. Thus, a large multiplex PCR can be performed and all exons separated on one gel.

8.2.3 Conformation sensitive gel electrophoresis (CSGE)

This technique is based on detection of heteroduplex DNAs by their aberrant migrations in mildly denaturing polyacrylamide gels (54). PCR amplified DNA is heteroduplexed with DNA from an unaffected individual, electrophoresed and visualized by ethidium bromide staining. Insertions and deletions of one or a few nucleotides cause bends in the DNA double helix, altering its gel migration. Single nucleotide substitutions cause the mismatched base to rotate out of the double helix, also causing a change in gel migration. This technique is used to detect the DNA

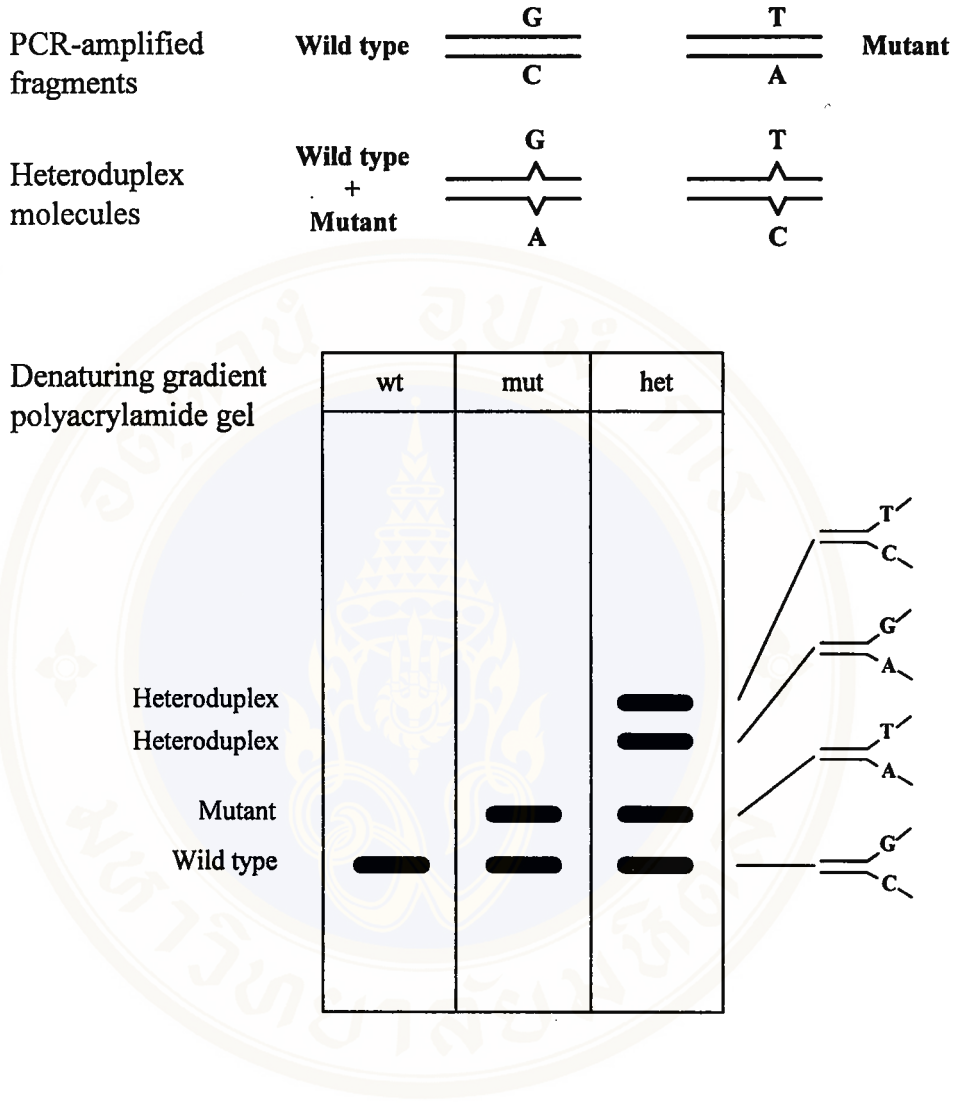


Figure 14. Principle of denaturing gradient gel electrophoresis (75).

Double stranded DNAs of wild type and mutant alleles as well as their heteroduplexes are run on denaturing gradient polyacrylamide gel. The less stable domains of DNA are melted at certain point of the denaturing gradient, making its mobility delayed. The wild type, mutant, and heteroduplex DNAs are melted and located at different positions.

fragments with the lengths from 200 to 800 bp. As sequence alterations in the terminal 50 bp may be missed, primers are extended well into intronic sequence or sequences overlapped to cover large exons. Advantages of the technique include its simplicity, high sample throughput, and lack of radiolabel requirement.

8.2.4 Chemical cleavage of mismatch (CCM)

Chemical cleavage of mismatch (CCM) is a method for detection of point mutations which depends upon the chemical modification and cleavage of mismatched bases in heteroduplex DNA (79). In this technique, radiolabeled DNA from an unaffected individual is mixed with excess patient DNA and the mixture denatured and re-annealed to produce heteroduplexes. Mismatched or unmatched bases occur at the sites of mutation. Hydroxylamine (HA) and osmium tetroxide (OsO_4) are used to modify the DNA at mismatched C and T bases, respectively, and the modified bases are subsequently cleaved by piperidine. Electrophoresis on denaturing polyacrylamide gels enables approximate location of the point of cleavage (Figure 15). CCM has the advantage, unlike most of other screening techniques, of indicating the location of a nucleotide change within a PCR fragment. It is also capable of scanning large DNA fragments of up to ~1.5 kb. The disadvantages of this technique are requiring the use of ^{32}P radiolabel, manipulation of many tubes, and toxic chemicals. The technique has been modified to allow greater sample throughput by using fluorescently labeled dUTP in place of radiolabeled primer, magnetic bead tagging of PCR product to reduce tube manipulations, and using an automated DNA sequencer to analyze screening results. Use of three different fluorescent dyes currently enables collection of three sets of data from each lane of the gel, making this a very efficient process (80).

8.2.5 Enzyme mismatch cleavage (EMC)

The principle of this technique is based on the sensitivity of mismatched bases to be cleaved by enzymes. The two most extensively used bacteriophage resolvases, T4 endonuclease VII (T4E7) and T7 endonuclease I (T7E1), have enabled the detection of mutations in DNA fragments of up to 1000 bp, with a sensitivity of well over 90% (82, 83). After PCR amplification of the mutant and target fragments, both products are subjected to a round of denaturation /renaturation to create heteroduplex molecules. The heteroduplexes are then incubated with one or the other enzyme. If mismatched bases are present within the heteroduplexes, the DNA will be cleaved by the resolvase near the mismatch site. The products are then separated by gel electrophoresis, and the site of the cleavage product indicates the approximate location of the mutation (Figure 15). However, the cleavage of heteroduplex DNA by the two resolvases does not occur precisely at the site of base mismatch; thus, a focused analysis of DNA sequence has to be performed to confirm the precise nature of the mutation at the indicated sites.

8.2.6 Ribonuclease (RNase) cleavage

This method involves the enzymatic cleavage of RNA at a single base mismatch in an RNA:RNA or RNA:DNA hybrid (84). A ribonucleic acid probe hybridized to RNA or DNA containing one base difference can be cut at the mismatch by ribonuclease. This allows mutations to be detected and located after analysis of the cleavage products by electrophoresis in a denaturing gel (Figure 15). The radioactive-labeled RNA probes are prepared by cloning with phage promoter (SP6 or T7), and the required enzymes are commercially available. The main problem with this method is that the sensitivity is not 100%, by using the only original RNase A (84); especially,

single base pair mismatches on the purine RNA probes as they are not cleaved efficiently under normal conditions. To improve the sensitivity, both sense and antisense are analyzed or specifically optimized RNases are used. These involve RNase A, RNase I, RNase T1, and RNase T2, and a combination of these enzymes. A recent technique of non-isotopic RNase cleavage assay (NIRCA); using cleavage of RNA:RNA heteroduplexes, can also improve the sensitivity (85).

8.2.7 Heteroduplex analysis (HA)

The heteroduplex involves spontaneous re-annealing of the single stranded PCR product in the latter stages of a PCR amplification. When amplifying from individuals heterozygous for any sequence difference, the single strands can re-hybridize exactly with the complementary strand or alternatively form a DNA hybrid (heteroduplex) with another variant. As a consequence the heteroduplex DNA has a region of at least one base pair mismatch, where a bulge or bend is generated. This structural feature produces different mobilities from the corresponding homoduplex without the mismatch. The resulting products are then separated by electrophoresis on non-denaturing gel (Figure 16). The heteroduplex system can also be used in combination with SSCP, due to the fact that the modes of detection of these two methods are very different and, hence, efficiency of detection can exceed 90% in DNA fragments from 250 to 400 bp (86). The products can be detected by silver staining which replaces radioisotopes. A recent development is an introduction of denaturing high-performance liquid chromatography (DHPLC) to analyze the heteroduplex (87). This method allows more precision and speed, and avoids the pouring of gels. Automation would allow high throughput without radioactivity but the high-performance liquid chromatography (HPLC) has to be included.

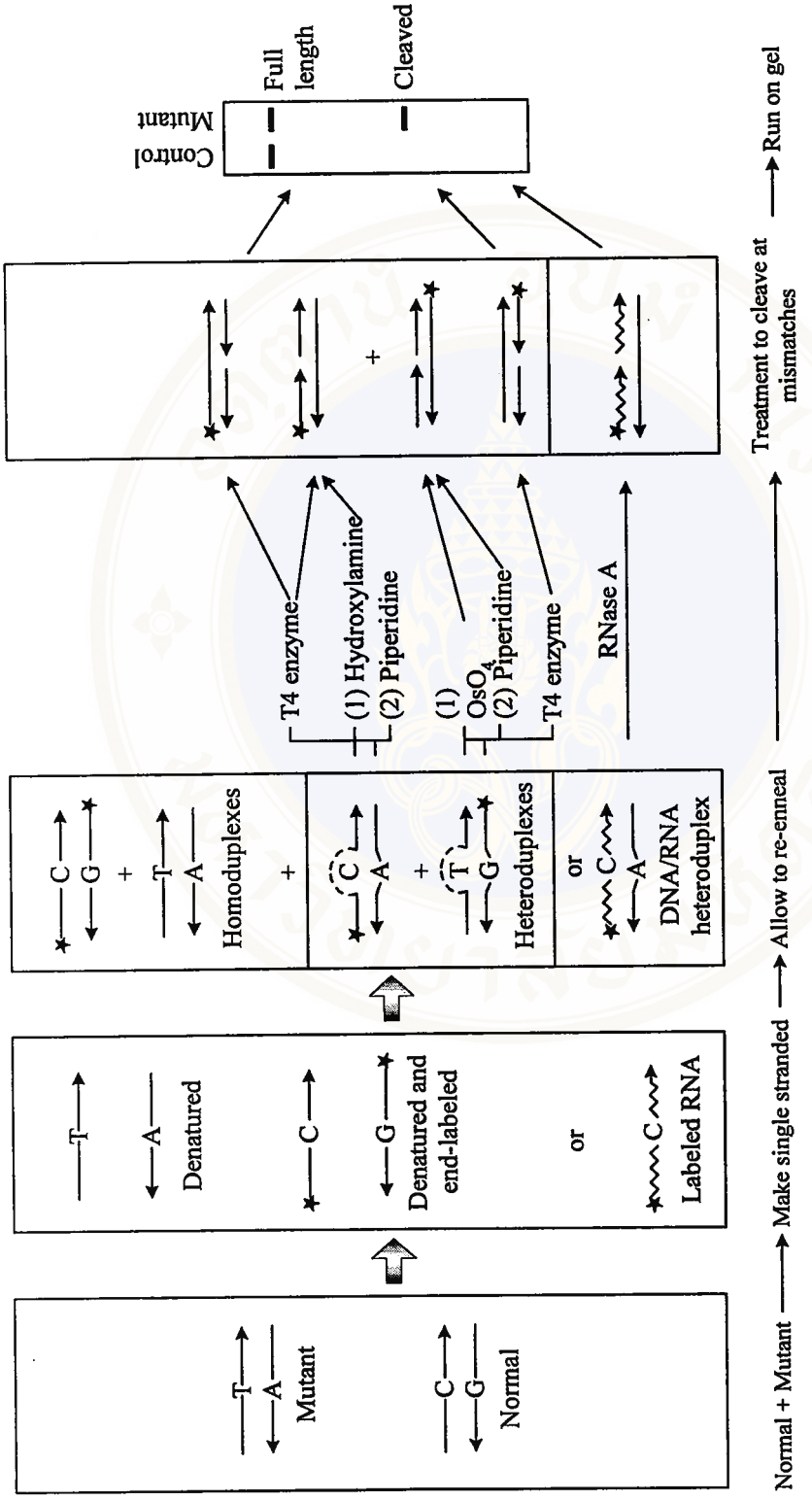


Figure 15. Cleavage of mismatch in heteroduplex (81).

The test sample is mixed with labeled wild-type sequence, denatured and allowed to renature. If the test sequence differs from the wild-type, heteroduplexes are formed which can be cleaved by chemical or enzymic treatment (see text). Cleavage is revealed by observing short labeled fragments on a gel.

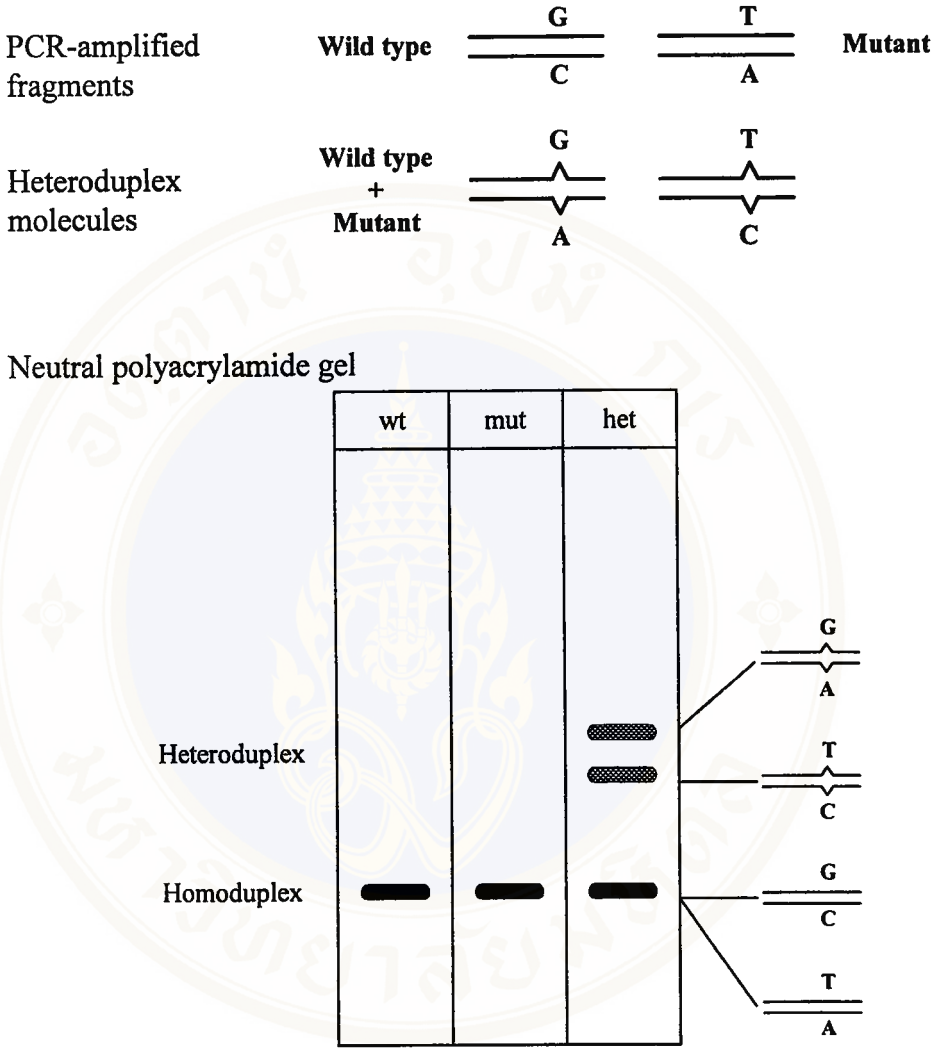


Figure 16. Principle of heteroduplex analysis (75).

The wild type and mutant amplified products can form heteroduplexes, which have different mobilities on non-denaturing polyacrylamide gel.

8.2.8 Dideoxy fingerprinting (ddF)

Dideoxy fingerprinting (ddF) is a hybrid between single-strand conformation polymorphism analysis (SSCP) and Sanger dideoxy sequencing. A Sanger sequencing reaction performed with only one dideoxy terminator is electrophoresed through a non-denaturing gel. The presence of a mutation can be detected as a result of gain or loss of a dideoxy termination segment (dideoxy component) and/or by a mobility shift of at least one of the termination segments that follow the mutation (SSCP component) (74). Since multiple segments can undergo a conformational change and indicate the mutation, ddF has a high redundancy and can detect virtually all mutations. ddF offers several obvious advantages over SSCP in providing information on the relative position of the sequence variant within the fragment being analyzed, and allows large PCR fragments to be generated once and analyzed in smaller subsegments by primer walking. It also reliably detects heterozygous mutations. This technique can be adapted to use fluorescence detection, which increases sensitivity of the procedure (88).

8.2.9 Protein truncation test (PTT)

The protein truncation test (PTT) is a specific test for frameshifts, splicing defects, or nonsense mutations, which truncate the protein product. PCR fragments, amplified from the gene or from its transcript, are used as templates in in *vitro* transcription/translation reactions to generate protein products. The protein product is then run on a gel. If the product is full length, no truncating mutation is present in the sequence. Truncating mutations result in shorter products, the size of which reveals the position of the mutation (70, 89) (Figure 17). The advantages of this method are that it allows screening of relatively large gene regions. Secondly, it only detects

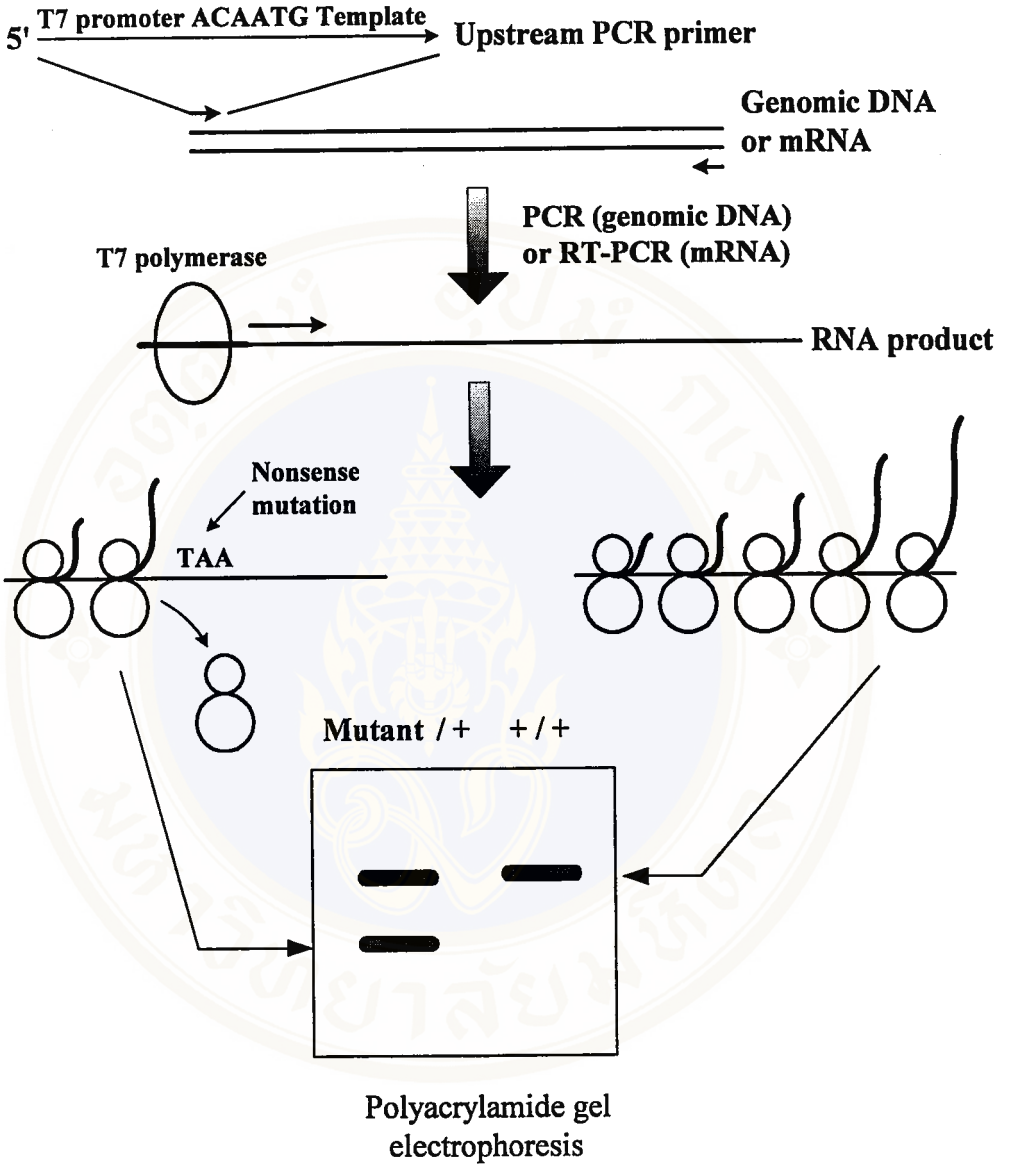


Figure 17. Principle of protein truncation test (90).

A part of gene or mRNA is amplified by PCR with the 5' primer containing T7 promoter and initiation codon (ATG). RNA is generated by *in vitro* transcription using T7 polymerase and used in cell-free translation to produce protein. The mutation that creates a stop codon will produce a truncated protein, which can be examined by polyacrylamide gel electrophoresis. (+) represents the wild type.

frameshift and nonsense mutations that are highly likely to be pathological; thus, polymorphic sequence variations will not be detected. Conversely, missense mutations are not detected by this method. A disadvantage is the need for RNA rather than DNA. This may present difficulties if gene transcripts cannot be obtained from easily obtainable tissues. Several variants of this method have been developed to give cleaner results, usually by incorporating an immunoprecipitation step (81).

8.2.10 DNA chips

DNA chips are a novel technology, which base on oligonucleotide arrays, and are touted to be able to analyze a large number of fragments in parallel (91). A massive array of oligonucleotides are attached onto a solid glass or silica support. The sequence of each oligonucleotide is the same as the wild type, except that the central base is systematically changed so that there is an oligonucleotide for every possible sequence variation. The test DNA is labeled with a fluorescent dye and preferentially hybridizes to the oligonucleotide in each set that exactly matches its sequence. A scanning confocal fluorescence microscope is used to record the result.

8.2.11 Nucleotide sequencing

Nucleotide sequencing is the gold standard for mutation detection. Sequencing a large gene for routine test is not a practicable proposition. However, this method is now generally applied semi-automatically and is very much more efficient than it was only a few years ago. It is possible therefore that sequencing genes to screen for mutations may become routine in the next few years (90).

CHAPTER IV

MATERIALS AND METHODS

MATERIALS

1. Blood samples

Blood samples were obtained from hemophilia A patients and family members who were attending at Hematology Clinics, Department of Pediatrics, Siriraj Hospital, Mahidol University, under the care of Dr. Chularatana Mahasandana and the departmental staffs. The patients were diagnosed as having hemophilia A based on clinical, hematological, and laboratory data. They were treated and followed up at this hospital for a long period of time. A total of 29 patients with moderate and severe degrees of hemophilia A were included in this study. Their factor VIII activities (FVIII:C) as determined by a one stage clotting assay (92) were ranged from <1% to 4%. Twenty-eight samples have previously been investigated for the factor VIII gene inversions by Southern blotting analysis. Ten samples from the patients with severe disease who were known to have gene inversion were subjected to be reinvestigated for the factor VIII gene inversion by the RT-PCR method developed (Table 3.1). Another 18 samples from the patients with severe and moderate disease who did not have gene inversion were examined for RNA defects (Table 3.2). Eight samples from this group were further screened for mutations by SSCP analysis (1/5, 7/3, 12/3, 13/3, 14/4, 14/5, 21/4, 22/2), while the remaining 10 samples were studies by another student. The last sample (76/3), which was not previously investigated for the factor VIII gene inversion by Southern blot analysis was examined for this defect by the RT-PCR method.

Table 3.1 Types of gene inversion and severities of disease in the hemophilia A patients included in the detection of factor VIII gene inversion by the RT-PCR method.

Patient ID	Family number	Types of inversion[1]	Severity
6/3	6	II	Severe
10/3	10	I	Severe
10/4	10	I	Severe
17/4	17	I	Severe
25/3	25	I	Severe
26/3	26	I	Severe
30/3	30	I	Severe
44/3	44	II	Severe
68/3	68	I	Severe
69/2	69	I	Severe
76/3	76	Unknown[2]	Severe

[1] Factor VIII gene inversion was determined by Southern blot analysis
Type I occurs from the intrachromosomal recombination between the F8A sequence in intron 22 and the most distal telomeric F8A sequence, while type II results from the similar recombination between the F8A sequence in intron 22 and the proximal telomeric F8A sequence.

[2] This case was not investigated for the factor VIII gene inversion by Southern blot analysis.

Table 3.2 Factor VIII activities and severities of disease in the hemophilia A patients included in RNA analysis and mutation screening.

Patient ID	Family number	Factor VIII activities (FVIII:C)[1]	Severity
1/5	1	<1%	severe
7/3	7	<1%	severe
8/3	8	<1%	severe
11/3	11	<1%	severe
12/3	12	<1%	severe
13/3	13	<1%	severe
14/4	14	2.5%[2]	severe
14/5	14	1.7%[2]	severe
19/3	19	<1%	severe
20/3	20	4%	moderate
21/4	21	2%	moderate
22/2	22	<1%	severe
24/3	24	1%[2]	severe
27/3	27	<1%	severe
28/3	28	3%	moderate
29/3	29	<1%	severe
33/3	33	<1%	severe
33/4	33	<1%	severe

[1] Factor VIII coagulant activity (FVIII:C) is expressed in U/dl.
[2] The severity of hemophilia A depends on both FVIII:C and clinical manifestations.

Twenty ml of blood samples were collected in EDTA for lymphocyte and other white blood cell (WBC) preparation. Details are described in the following method sections.

2. Instruments

Applied Biosystems 310 DNA sequencer version 3, ABI Inc., USA

Gene Amp PCR System 2400, Perkin Elmer Cetus, USA

Horizontal gel electrophoresis, Horizon 58, BRL, Life Technologies, USA

Polaroid camera, Fotodyne, USA

Transilluminator, TVC 312A, Spectronics, USA

UV-visible Spectrophotometer, Shimadzu UV-160A, Japan

3. Chemicals

Chemicals	Source
Acrylamide	Sigma
Ammonium persulphate	Sigma
Diethylpyrocarbonate (DEPC)	Promega
Ethylenediamine tetraacetic acid (EDTA)	Sigma
Formamide	Merck
Lymphoprep™	Nycomed Pharma AS
N,N'-methylene-bis-acrylamide	Sigma
Silver nitrate ($AgNO_3$)	Sigma
Sodium dodecylsulfate (SDS)	Sigma
N,N,N',N',-tetramethyl-ethylenediamine (TEMED)	BDH
TRIzol™ Reagent	Gibco BRL

The other chemicals and solvents were analytical grade and were purchased from Fluka, Gibco BRL, Merck, and Sigma.

4. Enzymes

Avian myeloblastosis virus reverse transcriptase (AMV-RT)	Promega
E-coli Ribonuclease H (RNase H)	Gibco BRL
ELONGase™ Enzyme Mix	Gibco BRL
Proteinase K	Gibco BRL
Recombinant ribonuclease inhibitor (rRNasin)	Promega
Restriction endonucleases:	Biolabs, Gibco BRL,
*Ava*II, *Bam*HI, *Bst*NI, *Dde*I, *Dra*I,	Boehringer Mannheim,
*Eco*RI, *Hae*III, *Hin*cII, *Hin*dIII, *Pst*I,	Promega, Stratagene
*Sau*3AI, *Sau*96I, *Sca*I, *Xcm*I, *Xba*I	
Taq DNA polymerase	Perkin Elmer

5. Oligonucleotide primers

The PCR primers used for amplification and sequencing of the factor VIIII gene were either previously described by Naylor *et al.*, 1991 (60), or designed by using the MacVector version 4.5.1 Program based on the factor VIII sequences (HUMF8C, Accession No. AH002692). Some general criteria in designing primers were:

1) primer length of 18-30 nucleotides,
2) G+C composition of 50 to 60% with random distribution,
3) lack of self-annealing regions within each primer,
4) no possibility of secondary structrue formation, and
5) no possibility of self annealing or annealing to each other.

The primer sets used for screening factor VIII gene inversion were designed to amplify regions covering the junction between exons 22 and 23, and at the 5'end of the gene. Names and locations of these primers, and the expected sizes of PCR products are also shown in Figure 18. The primers for screening the factor VIII gene inversion are listed in Table 4.1. The detail of primers used for long and overlapping amplification as well as DNA sequencing were shown in Table 4.2. Table 4.3 presents the detail of primers for allele-specific amplification (ASA) of the mutations characterized in this study.

6. Miscellaneous materials

Deoxynucleotide triphosphates	Promega
100 bp DNA Ladder	Gibco BRL
Phi X-174 RF DNA-*Hae* III digested	Biolabs
QIAquick Gel Extraction Kit	Qiagen
SuperScript™ Preamplification System for First Strand cDNA Synthesis	Gibco BRL
Low melting temperature agarose gel (SeaPlaque® Agarose)	FMC
ABI PRISM™ BigDye™ Terminator Cycle Sequencing Kit	PE Applied-Biosystems

7. Computer program

- Sequence Navigator software.
- MacVector™ 4.5.1 program (Kodak, Inc.).
- ClustalX (93) multiple sequence alignment program.

Table 4.1 Oligonucleotide primers for screening factor VIII gene inversion.

Site	Primer	Sequence (5'→3')	mRNA Position[a]	Tm (°C)
		Primers for internal control		
Outer primers				
5'Exon 1	F8Ex1L	ATGCTCTGCAAAGAAATTGGGAC	(-131)-(-109)	66
Exon 4	F8Ex4R	GGTCCACATGAGAAAGATATGAG	525-547	66
Inner primers				
Exon 1	F8Ex1L2	CTGCTTCTTTCTGTGCCTTTTG	21-42	64
Exon 4	F8Ex4R2	CATTGGACCATTCTCTTTCAGG	477-498	64
		Primers for exons 22-23 junction		
Outer primers				
Exons 20-21	F8Ex21L	TTCAGGACAATATGGACAGTGGG	6174-6196	68
Exon 25	F8Ex25R	CCATTCTGAAAAAAGAGAGTCCAC	6867-6890	68
Inner primers				
Exon 22	F8Ex22L	GATTATTCACGGCATCAAGACCC	6294-6316	68
Exon 23	F8Ex23R	CAACTCCATGCGAAGAGTGCTG	6534-6555	68

[a] Numbering is according to Wood *et al.* 1984 (23).

Table 4.2 Primers for PCR amplification and DNA sequencing of factor VIII gene.

Site	Primer	Sequence (5'→3')	mRNA Position[a]	Tm (°C)
5'Exon 1	F8Ex1L	ATGCTCTGCAAAGAAATTGGGAC	(-131)-(-109)	66
Exon 26	F8-3'R4	AAACCAGCAGGAAAATAAAAGAGC	8799-8822	66
5'Exon 1	F8N1A	GGGAGCTAAAGATATTTTAGAGAAG	(-70)-(-46)	68
Exon 9	F8N1B	TTCCTACCAATCCGCTGAGG	1303-1322	62
Exon 8	F8N2A	AGAAGCGGAAGACTATGATG	1080-1099	58
Exon 14	F8N2B	TTGCCTAGTGCTAGGGTGTC	2297-2316	62
Exon 14	F8N3A	GTCTATGGATTCTGGGGTGC	2114-2133	62
Exon 14	F8N3B	GGCAAAACTACATTCTCTTG	3673-3692	56
Exon 14	F8N4A	CGTAGGACTCAAAGAGATGG	3537-3556	60
Exon 14	F8N4B	CTGTTTCTTAGAACATGTGG	5200-5219	56
Exon 14	F8N5A	ACCCACCAGTCTTGAAACGC	4973-4992	62
Exon 20	F8N5B	TCCATATTGTCCTGAAGCTG	6170-6189	58
Exon 18	F8N6A	TTCATTTCAGTGGACATGTG	5924-5943	56
Exon 26	F8N6B	GGCTTCAAGGCAGTGTCTGC	7158-7177	64
Exon 26	F8Ex26L	CACACCTGTGGTGAACTCTCTAG	6927-6949	70
Exon 26	F8-3'R3	CCTGCATCATTTGTGGATTGTGAC	8564-8587	70
5'Exon 1	F8N1C	GAGAAGAATTAACCTTTTGCTTCTC	(-51)-(-27)	68
Exon 8	F8N1D	CAGCAGCAATGTAATGTACC	1203-1222	58
Exon 8	F8N2C	TCTGAAATGGATGTGGTCAG	1114-1133	58
Exon 14	F8N2D	AAGCTTCTTGGTTCAATGGC	2263-2282	58
Exon14	F8N5C	TCTTCAGTCAGATCAAGAGG	5019-5038	58
Exon 20	F8N5D	CTCTAATGTGTCCAGAAGCC	6138-6157	60

[a] Numbering is according to Wood *et al.* 1984 (23).

Table 4.2 Primers for PCR amplification and DNA sequencing of factor VIII gene (continued).

Site	Primer	Sequence (5'→3')	mRNA Position[a]	Tm (°C)
Exon 19	F8N6C	ATCTACATGCTGGGATGAGC	6071-6090	60
Exon 26	F8N6D	AAGGTAGAAGGCAAGCCAGG	7124-7143	62
Exon 14	5A.1	CCCGCAGCTTTCAAAAGAAAACAC	5120-5143	70
Exon 16	5B.1	CAGGCTTTGCAGTCAAACTCATC	5539-5561	68
Exon 4	F8Ex4L	CAGTCAAAGGGAGAAAGAAGATG	411-433	66
IVS 14	F8Ex15F1	GGATGTGAGGCATTTCTACC	(-67)-(-48) of 5'Ex 15	60
IVS 15	F8Ex15B1	GGGAATACATTATAGTCAGCAAG	(+53)-(+75) of 3'Ex 15	64
IVS 15	F8Ex16F1	GGATGTAAACCCTAAGGACC	(-76)-(-57) of 5'Ex 16	60
IVS 16	F8Ex16B1	TTAAACCAAAAAGTGGTCAGC	(+20)-(+40) of 3'Ex 16	58

[a] Numbering is according to Wood *et al.* 1984 (23).

Table 4.3 Primers for allele specific amplification (ASA) of mutations characterized in this study.

Site	Primer	Sequence (5'→3')	mRNA Position[a]	Tm (°C)
IVS 14	F8Ex15F1	GGATGTGAGGCATTTCTACC	(-67)-(-48) of 5'Ex 15	60
IVS 15	ASAEx15Wt	TTCCACTGTCCCTAACGCAC[b]	(+1)-(+20) of 3'Ex 15	62
IVS 15	ASAEx15Mt	TTCCACTGTCCCTAACCCAA	(+1)-(+20) of 3'Ex 15	60
Exon 26	F8-3'L3	AACACTCCAGTCTGCCATATCACC	8196-8219	72
Exon 26	F8-3'R3	CCTGCATCATTTGTGGATTGTGAC	8564-8587	70
IVS 5	F8Ex6F1	CATGAGACACCATGCTTAGCTGAC	(-49)-(-28) of 5'Ex 6	66
Exon 6	ASAEx6Wt	GACCTGTTTACATAACTATTGAATG	755-779	66
Exon 6	ASAEx6Mt	GACCTGTTTACATAATCATTGAATA	755-779	64
Exon 14	F8Ex14L	ACAGCCTGTCTGAAATGACACAC	2543-2565	70
Exon 14	F8Ex14R	CATACTTGGGGGTCCTAAGGAAC	2762-2784	70

[a] Numbering is according to Wood *et al.* 1984 (23).
[b] The underlined base at the 3' terminus is the base complementary that of each allele. The underlined bases at the positions 3rd or 4th, and 9th or 10th are additional mismatching bases, deliberately introduced into the primers to increase specificity of ASA.

METHODS

1. Preparation of lymphocytes from peripheral blood

Peripheral lymphocytes were isolated from fresh blood by centrifugation onto a Ficoll cushion. Blood sample (20 ml) was diluted with equal volume of phosphate buffer saline (PBS; 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.4) before carefully overlaying onto 4 tubes of 4 ml each of Lymphoprep™. The samples were then centrifuged at 2,000 rpm, 4 °C for 30 min. The white ring containing lymphocytes was collected to a fresh tube. The lymphocytes were washed with 10 ml of PBS and centrifuged at 1,500 rpm, 4 °C for 10 min. The supernatant was discarded and the cells were washed again before collecting the cell pellet for RNA preparation.

2. Preparation of total RNA

Total RNA was isolated from peripheral blood lymphocytes by using a commercial modification of single-step acid guanidinium thiocyanate-phenol-chloroform extraction method. Five hundred microliters of TRIzol™ reagent were added to lymphocytes. The mixture was then mixed vigorously until homogeneous and placed at room temperature for 5 min. After adding 100 µl of chloroform, the mixture was mixed, placed at room temperature for 2-3 min and centrifuged at 12,000xg, 4 °C for 15 min. The aqueous phase was transferred to a fresh tube, mixed with 250 µl of isopropanol, and then placed at room temperature for 15 min to precipitate RNA. The mixture was centrifuged at 12,000xg, 4 °C for 10 min. The supernatant was discarded. RNA pellet was washed with 500 µl of 75% ethanol, mixed, and centrifuged at 7,500xg, 4 °C for 5 min. The ethanol was then discarded

and RNA pellet was dried at room temperature for 5-10 min. The pellet was subsequently dissolved in 10-20 µl of DEPC-treated water. The optical density (OD) of 1:50 diluted RNA solution was measured at wavelength 260 and 280 nm. The OD260/OD280 ratio was calculated to estimate RNA purity (the absorbance ratio should be 1.8-2.0) and its original concentration (ng/µl) was determined from the formula: OD260 x 40 x 50. The RNA solution was further used or kept at -70 °C.

3. Preparation of genomic DNA

After preparing lymphocytes, the blood cells in the bottom layer of Lymphoprep™ were collected to separate other white blood cells. An equal volume of RBC-lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.1) was added, and mixed homogeneously. The suspension was then left at room temperature for 5 min and centrifuged at 2,000 rpm for 10 min. The RBC lysate was discarded and the white blood cell pellet was collected. The same procedure was repeated 2-3 times or until the pellet was pale. The pellet was collected after final washing with PBS by centrifugation at the condition previously described and then kept at -20 °C for genomic DNA preparation.

For DNA preparation, 4 ml of 20 mM Tris-HCl pH 7.5 and 5 mM EDTA (TE 20-5) solution were added into the WBC pellet. The cell suspension was mixed vigorously until it became homogeneous. After adding 200 µl of 10% SDS and 200 µl of 2 mg/ml proteinase K, the mixture was incubated at 37 °C overnight. The mixture was gently extracted twice with 1/2 volume of phenol (equilibrated with 0.1 M Tris-HCl pH 8.0 and 0.1% 8-hydroxyquinoleine) and 1/2 volume of chloroform-isoamyl alcohol (24:1) to remove digested proteins from DNA solution. In each step, the

mixture was centrifuged at 2,500 rpm for 10 min, and the organic phase in the bottom layer was discarded. The washing and centrifugation step were repeated twice more with one volume of chloroform-isoamyl alcohol (24:1). DNA was precipitated out of aqueous phase by adding 1/10 volume of 4 M NaCl and 2 volumes of chilled absolute ethanol. After collection of DNA pellet by centrifugation at 3,000 rpm for 10 min, it was washed once with 5 ml of 70% ethanol, and centrifuged again to obtain the final pellet. The air-dried DNA pellet was dissolved in 0.5-1.0 ml of sterile distilled water. The DNA concentration was determined from OD measurement of 1/50-1/100 diluted DNA at 260 and 280 nm. The OD260/OD280 ratio was calculated to verify purity of DNA and its concentration (in μg/ml) was estimated by formula: OD260 x 50 x dilution factor.

4. Detection of factor VIII gene inversion by RT-PCR

The method for screening factor VIII gene inversion was performed by cDNA synthesis from mRNA followed by PCR. The PCR reaction was performed in two regions, at 5' of gene (for internal control) and around the junction between exon 22 and exon 23 (for gene inversion detection) as shown in Figure 18. The normal cDNA could be amplified in the latter region while the cDNA from the factor VIIII gene inversion could not. The initial reaction mixture (10 μl) contained 200 ng of RNA, 1 μl of 10xPCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2 μl of 25 mM $MgCl_2$, 10 pmole each of F8Ex4R and F8Ex25R primers, and 1 mM nucleotides. The reaction mixture was incubated at 70 °C to destroy secondary structure of RNA for 10 min and cooled on ice for 5 min before adding 20 U of RNasin® Ribonuclease inhibitor, 4 U of AMV-RT. Then, the mixture was incubated at 37 °C for 1 hour for

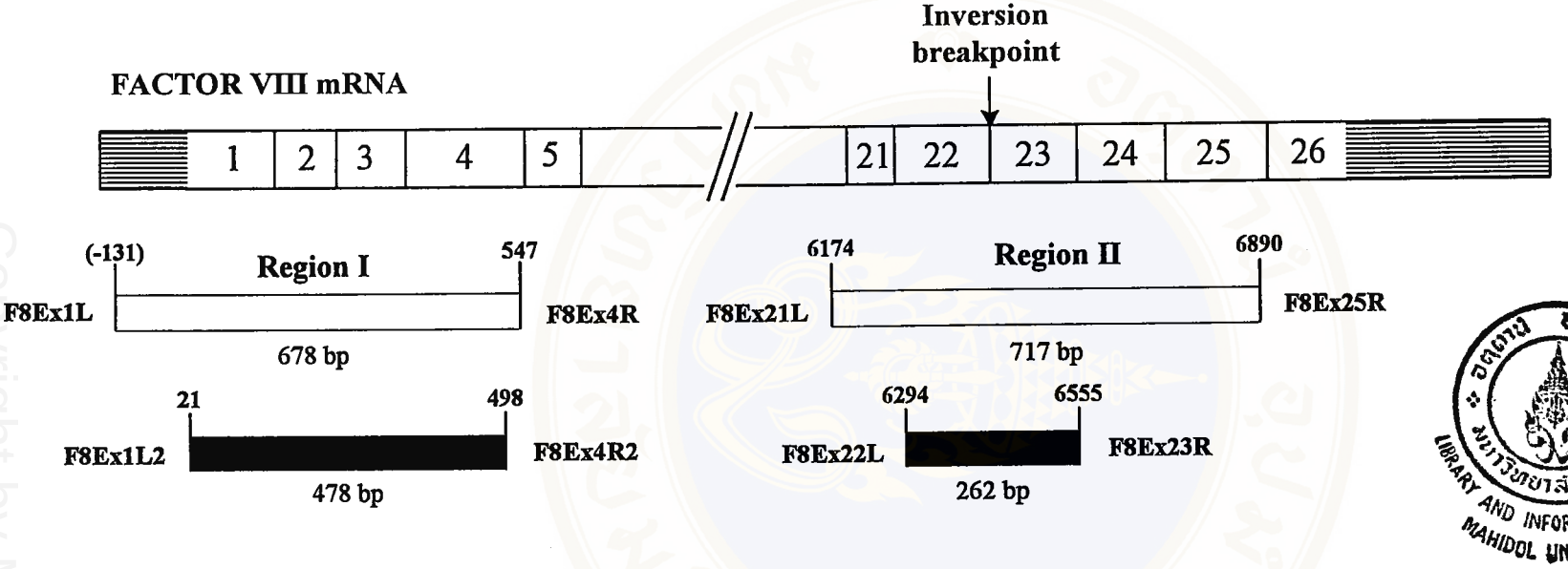


Figure 18. Strategy for detection of factor VIII gene inversion.

Diagram of the factorVIII mRNA with exon junctions represented by vertical lines is shown. The arrow indicates the inversion breakpoint. Region I is the region for the internal control amplification, and Region II is the region for investigation of gene inversion of which mRNA abnormality prevents the RT-PCR amplification across the boundary between exons 22 and 23. Open boxes represent the primary PCR product, and black boxes represent the nested PCR product.

cDNA synthesis. For the 50 µl amplification reaction, cDNA synthesis mixture (10 µl), 4 µl of 10xPCR buffer (100 mM Tris-HCl, pH 8.3, and 500 mM KCl), 4 µl of 25 mM $MgCl_2$, 10 pmole each of F8Ex1L and F8Ex21L primers, and 1.25 U *Taq* DNA polymerase were mixed together. PCR was performed on a Gene-Amp PCR system 2400. Following an initial denaturation at 94 °C for 30 s, thermocycling of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 1 min was carried out for a total of 40 cycles. For nested PCR, the amplification reaction was carried out using 2 µl of RT-PCR product as a template in 25 µl of volume consisting of 10 pmole each of F8Ex1L2, F8Ex4R2, F8Ex22L, and F8Ex23R primers, 1.5 mM $MgCl_2$, 0.2 mM nucleotides, and 0.625 U *Taq* DNA polymerase in 10 mM Tris-HCl, pH 8.3, and 50 mM KCl buffer. Thirty-five temperature cycles were carried out. Each cycle consists of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s. The PCR products were electrophoresed on 2% agarose gel in 1x TAE (40 mM Tris-acetate, pH 8.0, and 1 mM EDTA) at 100 volts for 25 min. The primers used in this experiment are listed in Table 4.1.

5. Amplification of factor VIII cDNA

5.1 Amplification of coding sequence of factor VIII gene by long RT-PCR

RNA was reverse transcribed into cDNA by using oligo$(dT)_{12-18}$ primer and SuperScript™ Preamplification System kit, according to the instruction manual. The PCR primers were designed to amplify full length of the factor VIII coding sequence (exon 1-26) (Table 4.2). The PCR fragment was 8,953 bp in length. The initial reaction mixture (12 µl) containing 5 µg of total RNA, 0.5 µg oligo$(dT)_{12-18}$, and 30 U of RNasin® Ribonuclease inhibitor, was incubated at 70°C to destroy the secondary

structure of RNA for 10 min and cooled on ice for at least 1 min before adding 2 µl of 10xPCR buffer (100 mM Tris-HCl, pH 8.3, and 500 mM KCl), 2 µl of 25 mM $MgCl_2$, 2 µl of 0.1M DTT, and 1 µl of 10 mM nucleotides. The reaction mixture was pre-incubated at 42°C for 5 min before adding 200 U of Superscript™ II reverse transcriptase and further incubated for 50 min for cDNA synthesis from mRNA. The reaction was terminated by incubating at 70 °C for 15 min before adding 2 U of RNase H in order to remove RNA template and incubated at 37 °C for 20 min. Finally, the reaction containing cDNA was kept at -20 °C or used as template to amplify long PCR product by using ELONGASE enzyme mix and two specific primers, F8Ex1L and F8-3'R4 (sequences and positions of these primers were presented in Table 4.2 and Figure 19). The PCR reaction was carried out in a total volume of 50 µl containing 2 µl of cDNA synthesis reaction, 5 µl of 5x buffer A (300 mM Tris-SO_4, pH 9.1, 90 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$), 5 µl of 5x buffer B (300 mM Tris-SO_4, pH 9.1, 90 mM $(NH_4)_2SO_4$, 10 mM $MgSO_4$), 0.2 mM nucleotides, 10 pmole of each primer, and 2 U of ELONGASE enzyme mix. A 30-cycle PCR was performed after heating at 94 °C for 1 min; each cycle contained denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 68 °C for 10 min.

5.2 Nested amplification of overlapping fragments

Nested PCRs were performed by using 7 primer pairs, designated as F8N1A/F8N1B, F8N2A/F8N2B, F8N3A/F8N3B, F8N4A/F8N4B, F8N5A/F8N5B, F8N6A/F8N6B, and F8Ex26L/F8-3'R3, to generate 7 overlapping fragments with the size of 1,392 bp, 1,237 bp, 1,579 bp, 1,683 bp, 1,217 bp, 1,254 bp and 1,661 bp, respectively (Figure 19). The PCR reaction was carried out in a total volume of 25 µl

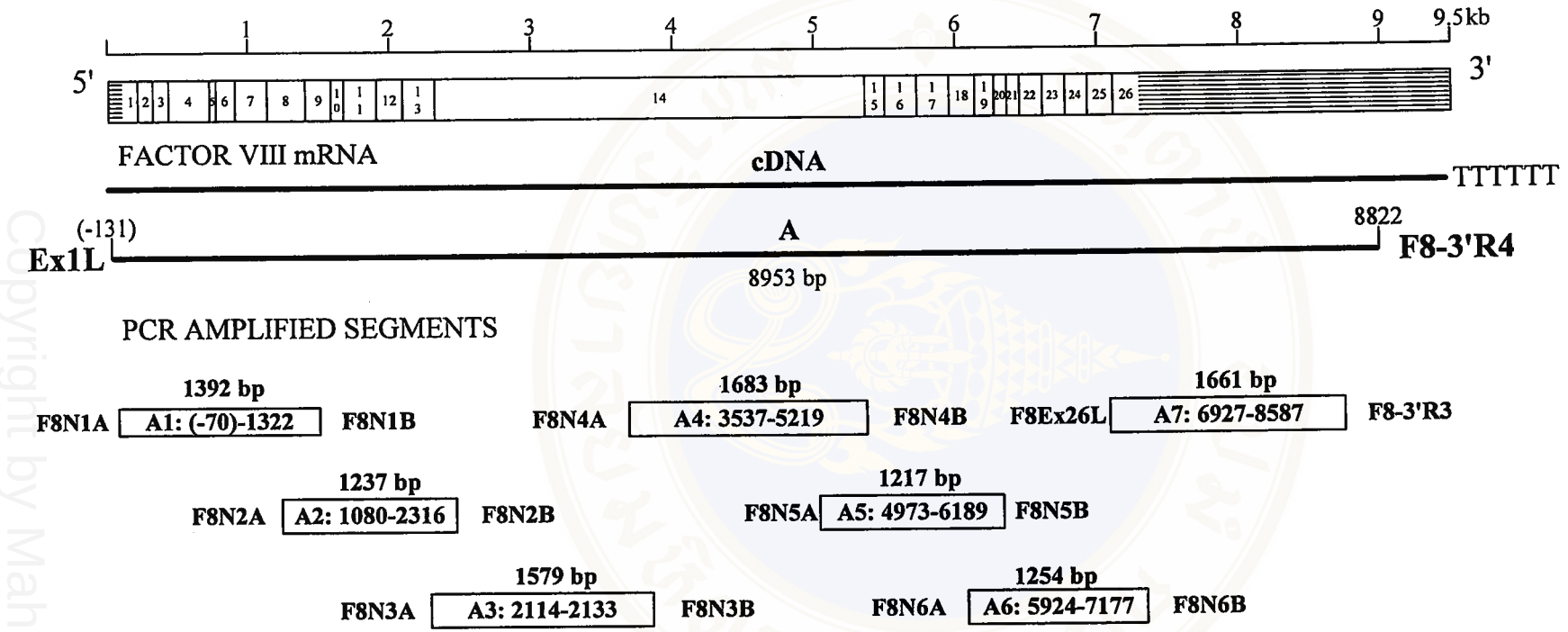


Figure 19. Strategy for amplifications of 7 overlapping fragments from factor VIII mRNA containing entire coding sequence.

A schematic representation of factor VIII mRNA, cDNA, and nested DNA fragments amplified by using Ex1L/ Ex3'R4 primers and nested primers (F8N1A/F8N1B, F8N2A/F8N2B, F8N3A/F8N3B, F8N4A/F8N4B, F8N5A/ F8N5B, F8N6A/F8N6B, and F8Ex26L/F8-3'R3

by adding 1 µl of long PCR product, 10 pmole of each primer, 0.2 mM nucleotides, 1.5 mM $MgCl_2$, and 1.25 U *Taq* DNA polymerase in PCR buffer containing 10 mM Tris-HCl, pH 8.3, and 50 mM KCl. After mixing and spinning briefly, PCR was performed on Gene-Amp PCR system 2400. It was started with denaturation at 94 °C for 1 min followed by 30 cycles consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 68 °C for 3 min, respectively. After completing the amplification with an extension at 68 °C for 7 min, the quantity and quality of amplification products were checked by electrophoresis on 1% agarose gel in 1x TAE (40 mM Tris-acetate, pH 8.0, and 1 mM EDTA) and ethidium bromide staining.

In some occasions, the second round of nested PCR was performed to further amplified 4 fragments generated by amplifications with F8N1A/F8N1B, F8N2A/F8N2B, F8N5A/F8N5B, F8N6A/F8N6B by using another 4 primer pairs, namely F8N1C/F8N1D, F8N2C/F8N2D, F8N5C/F8N5D, and F8N6C/F8N6D. The sizes of fragments were 1,273 bp, 1,169 bp, 1,139 bp, and 1,073 bp, respectively. The reaction mixture and process were the same as mentioned above.

6. Amplification of factor VIII genomic DNA

The exon 14 region was also amplified by using genomic DNA as template. Two overlapping fragments with the size of 1,579 bp, 1,683 bp were amplified by two sets of primers, F8N3A/F8N3B and F8N4A/F8N4B, respectively. Approximate 150-300 ng of genomic DNA was added with 10 pmole of each primer pair in a 25 µl reaction volume. The final concentrations of the reagents in the solution were as follows: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM nucleotides, and 1.25 U *Taq* DNA polymerase. The DNA was denatured at 94 °C for 2 min after which 30 cycles of PCR were performed; each cycle consists of denaturation at 94 °C for 30 s,

annealing at 55-60 °C for 30 s, and extension at 72 °C for 3 min. The amplified products were checked by electrophoresis on 1% agarose gel and ethidium bromide staining.

7. Agarose gel electrophoresis

The amplified DNA obtained from PCR was examined by agarose gel electrophoresis. Seven microliters of amplified DNA were mixed with 3 µl of 6x loading buffer (30% glycerol in water, 0.25% bromo-phenol blue, and 0.25% xylene cyanol FF). One hundred base-pair DNA ladder was used as a standard marker for estimation of molecular sizes of DNA fragments. The electrophoresis was performed on 1% agarose gel in 1x TAE buffer (40 mM Tris-acetate, pH 8.0, and 1 mM EDTA) at constant voltage 100 volts for 40 min. The agarose gel was stained with 0.5% ethidium bromide, destained in distilled water, visualized on a UV transilluminator, and photographed by a polaroid camera (Fotodyne).

8. Phenol-chloroform extraction of PCR product

After PCR amplification and checking, the remaining product was purified by phenol-chloroform extraction before performing restriction enzyme digestion. The PCR product was transferred into a 1.5 ml microfuge tube, and filled with TE 10-1 buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, pH 7.8) until the total volume was 200 µl. The mixture was added with equal volume of phenol and chloroform mixture (1:1), mixed until the emulsion formed, and then centrifuged at 12,000 rpm for 3 min. The aqueous phase was transferred to a fresh tube. The remaining (organic phase and interface) were extracted again by adding with 150 µl TE 10-1 buffer, mixed and centrifuged at 12,000 rpm for 3 min. The second aqueous phase was collected

together with the first one. DNA was precipitated out of aqueous phase by adding with 1/10 volume of 3 M sodium acetate and 3 volumes of chilled absolute ethanol. The mixture was then placed at -20 °C overnight to enhance DNA precipitation. After centrifugation at 12,000 rpm for 10 min, the supernatant was discarded. The pellet was washed with 70% ethanol and left to dry at room temperature. DNA pellet was dissolved in 30-50 µl of distilled water.

9. Restriction enzyme digestion of PCR product

Prior to mutation screening by SSCP, the purified PCR product was digested with various restriction enzymes as shown in Table 6. The digestion condition was performed according to the manufacturer's instructions. The reaction was generally carried out in total volume of 25 µl consisting of 10 µl of purified PCR product and 10 units of restriction enzyme in a recommended buffer. The reaction mixture was incubated at an appropriate temperature and incubation time to obtain the complete digestion.

10. Screening mutation of the factor VIII gene by single strand conformation polymorphism (SSCP) and silver staining

10.1 Polyacrylamide gel preparation

To prepare 10% polyacrylamide with 5% glycerol, 2.5 ml of 40% stock of acrylamide : bisacrylamide (49:1, w/w) solution, 2 ml of 5x TBE buffer, 0.5 ml of glycerol, 4.95 ml of distilled water, 70 µl of 10% ammonium persulphate, and 4 µl of TEMED were mixed. The polyacrylamide gel was poured into the assembled glass plates and allowed to polymerize for about 1 hour.

10.2 Electrophoresis

Two microliters of PCR product were mixed with 8 µl of loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol FF, 20 mM EDTA, 10 mM NaOH) before denaturation at 95 °C for 10 min and immediately cooled on ice. A double-stranded DNA control was prepared in the same way by using loading buffer without NaOH and the sample was not pre-heated before loading to the gel. The sample was loaded onto the gel (Mini-Protean II Electrophoresis Cell, Bio-Rad) and electrophoresed in 1x TBE buffer at constant 20 mA, room temperature for 2-6 hours depending on the size of DNA fragment. The SSCP patterns were visualized by silver staining.

10.3 Silver staining

After electrophoresis, the gel was removed from the electrophoretic set and stained by silver-staining method. The gel was fixed with 40% methanol for 10 min. The methanol was discarded before soaking with 160 mM HNO_3 for 6 min. The gel was rinsed with de-ionized water once and washed again for 5 min. Staining of the gel was performed for 20 min using 0.2% $AgNO_3$. Rinsing and washing with de-ionized water for 5 min were conducted after discarding silver nitrate solution. Developer solution containing 3% Na_2CO_3 and 0.05% formaldehyde was added and mixed until DNA bands appeared. The developing reaction was stopped by addition and incubation with 0.1 M citric acid for 5 min. The gel was rinsed and washed with de-ionized water and subjected to air-drying inside 2 cellophane sheets for permanent record.

11. Identification of mutation by automated DNA sequencing

The PCR product of interest was purified from low melting temperature agarose gel after eletrophoresis, by using the QIAquick™ Gel Extraction Kit, following the manufacturer's instructions. To identify mutation by automated DNA sequencing, the purified PCR product was sequenced by an automated sequencer ABI-PRISM™ 310. The sequencing reaction was performed by using ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit, according to the manufacturer's instruction. The reaction mixture was made by mixing 4 µl terminator ready reaction mix, 3.2 pmol primer, 30-40 ng purified PCR product and distilled water to the volume of 20 µl. The cycle sequencing was carried out in the GeneAmp PCR System 2400, by running 25 cycles consisting of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. After that, the sequencing product was precipitated from the excessive dye terminator by adding 2 µl of 3M sodium acetate, pH 4.6 and 50 µl of 95% ethanol. The pellet was washed with 250 µl of 70% ethanol, centrifuged, and finally dried for 10-15 minutes in the dark place. The dried pellet was resuspended in 25 µl template suppression reagent (TSR), heated at 95°C for 2 minutes to denature the sequencing product, chilled on ice and loading to automated DNA sequencer ABI-PRISM™310. Fluorescent signals were detected and recorded by a computer using Data Collection software. Determination and analysis of nucleotide sequence were performed by Sequence Navigator software and MacVector™ 4.5.1 program.

CHAPTER V

Results

1. Detection of factor VIII gene inversion by RT-PCR method

A single crossing over event causes a DNA inversion of roughly 600 kb, resulting in the factor VIII gene being divided into two parts, exons 1-22 and exons 23-26, which are widely separated in opposite directions. RT-PCR method, was developed for screening the factor VIII gene inversion. In this study, 11 patients were examined for the gene inversion, ten of them were known to have the gene inversion by Southern blot hybridization and the other was not known. RNA was taken to analyze by RT-PCR and nested PCR (section 4 of the Methods). The PCR products were electrophoresed on agarose gel and stained with ethidium bromide. In normal sample, there were two PCR products with the sizes of 478 bp (internal control) and 262 bp (the region between exons 22 and 23). The sample with the gene inversion in region between exons 22 and 23 showed only the product with the size of 478 bp, without that of 262 bp. Figure 20 shows the result that the normal sample had 2 bands and the patients' samples with the gene inversion had only 1 band of the internal control. The last sample (lane 13), which was not previously known for the gene inversion, showed only the band of internal control indicating that this sample carried the gene inversion.

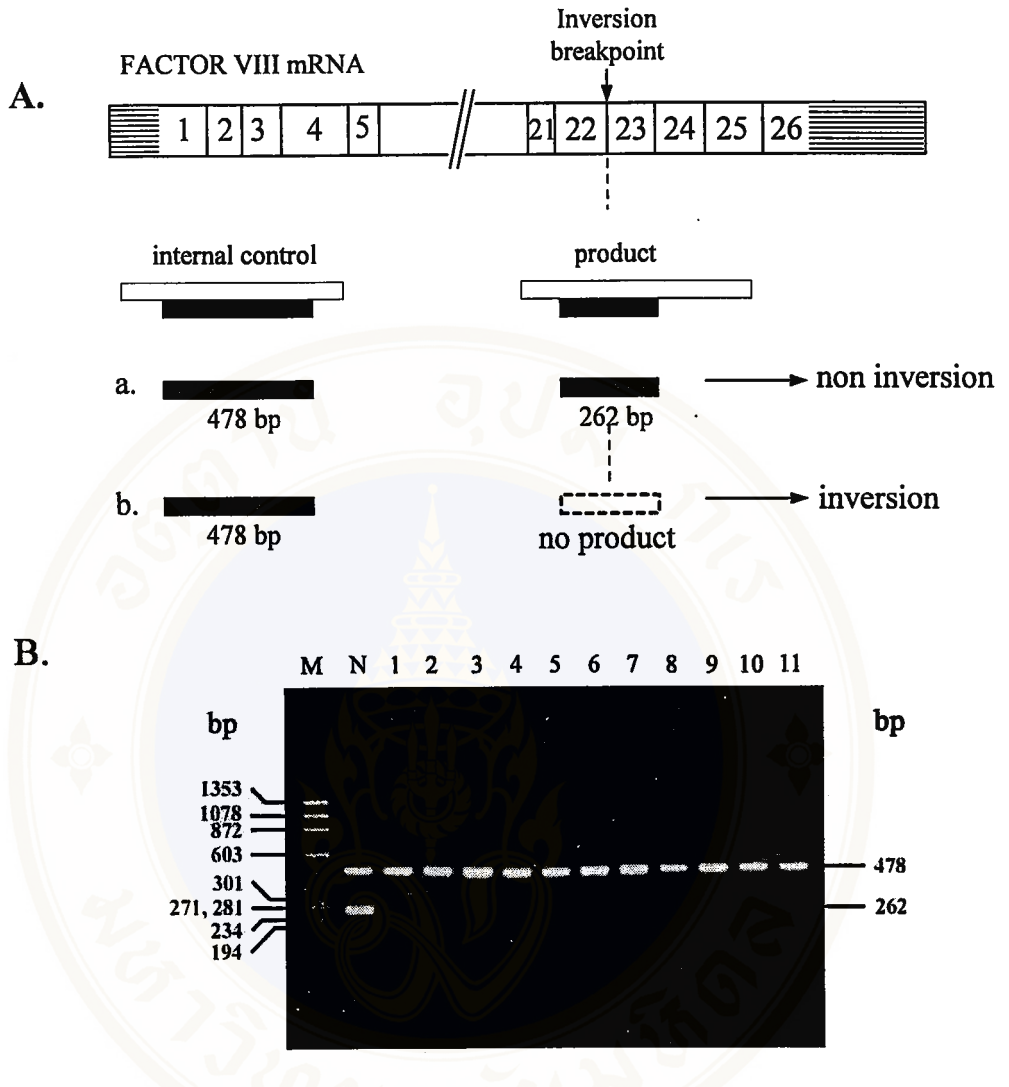


Figure 20. Detection of factor VIII gene inversion.

A. Diagram of factor VIII mRNA showing the region of inversion breakpoint and two-stage (nested) amplifications. Open boxs represent the primary PCR product, and black boxs represent the nested PCR product. In the case of non-inversion (a), there are two products of internal control (expanding from exon 1 to 4), and test product of exon 22/23 boundary. In the case of inversion (b), there is only one product of internal control.

B. Agarose gel electrophoresis of amplified cDNAs from 11 severe hemophilia A patients for detection of factor VIII gene inversion. M = *Hae*III-digested PhiX174RF DNA markers; N = normal individual; 1-11 are patients'samples. Lane11 is a new patient who was not previously known for the gene inversion. The normal sample showed PCR product of 478 bp and 262 bp in sizes but the patients'sample that had the gene inversion showed only a PCR product of 478 bp in size.

2. Amplification of factor VIII cDNA

RNA samples from hemophilia A patients without the gene inversion were reverse transcribed to be cDNA and amplified to obtain the entire coding sequence of the factor VIII gene before further amplifications to generate seven overlapping PCR fragments (Figure 21). Five overlapping fragments designated as fragments A1, A2, A5, A6, and A7 were generated by amplified of factor VIII cDNA using F8N1A/F8N1B, F8N2A/F8N2B, F8N5A/F8N5B, F8N6A/F8N6B, and F8Ex26L/F83'R3 primers, respectively (section 5.2 of the Methods). The nucleotide positions of these fragments were, A1: (-70) to1322, A2: 1080 to 2316, A5: 4973 to 6189, A6: 5924 to 7177, and A7: 6927 to 8587. The large exon 14 was amplified in two fragments, designated as A3 and A4, either by using either long cDNA or genomic DNA as the template by using F8N3A/F8N3B, F8N4A/F8N4B primers (sections 5.2 and 6 of the Methods). Their nucleotide positions were A3: 2114 to 3692, and A4: 3537 to 5219. Each fragment of the PCR products was separately electrophoresed on 1% agarose gel (section 7 of the Methods). Figure 22 (A to G) shows the results of nested PCR amplifications of the 7 fragments of factor VIII cDNA from 18 hemophilia A patients. Almost all of them showed correct size for each set of overlapping fragments. However, it was found that one patient's sample had a shorter size of the A5 fragment (Figure 22 E, lane 3). This fragment was further analyzed in details.

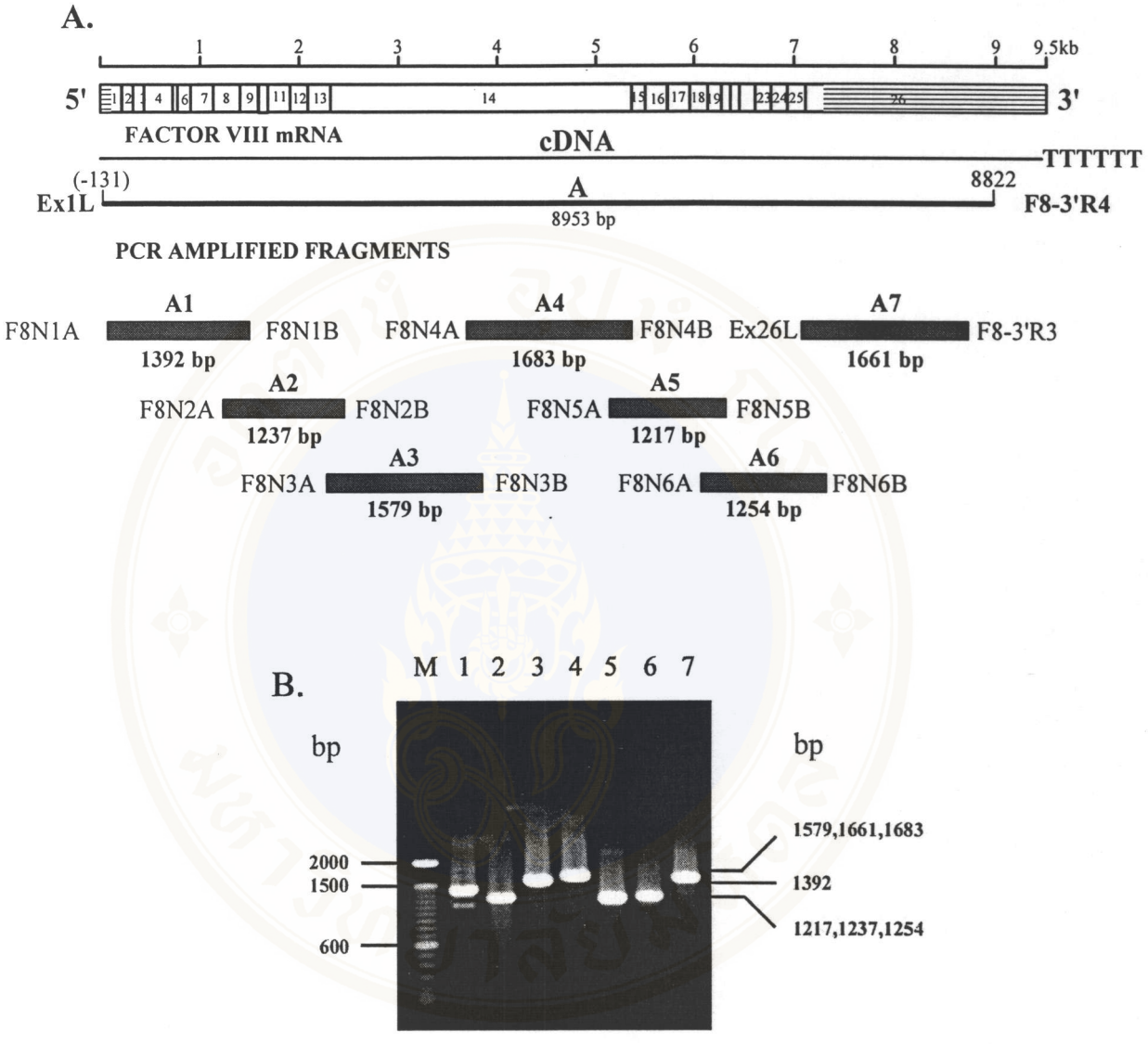
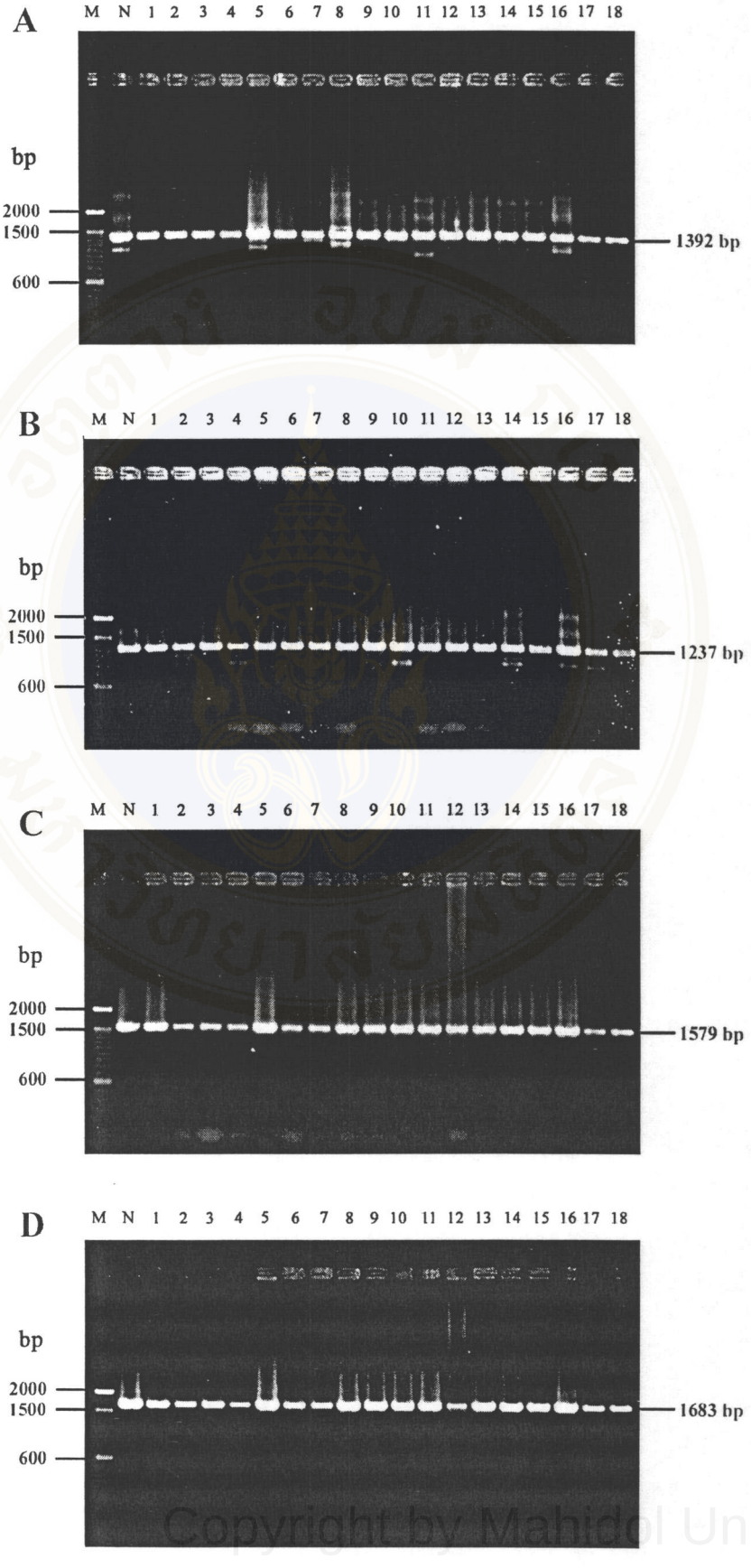


Figure 21. Factor VIII cDNA synthesis, long PCR, and nested PCR.

A. Diagram of the factor VIII mRNA with exon junctions represented by vertical lines and 5' and 3' untranslated regions by shaded areas. Fragments generated by cDNA synthesis, long PCR, and nested PCR are shown underneath.

B. PCR products of nested amplifications of factor VIII cDNA detected by 1% agarose gel electrophoresis in 1x TAE buffer. Lane M is 100 bp DNA markers. Lanes 1-7 are nested PCR products, fragments A1-A7, with the sizes of 1392, 1237, 1579, 1683, 1217, 1254, and 1661 bp, respectively.



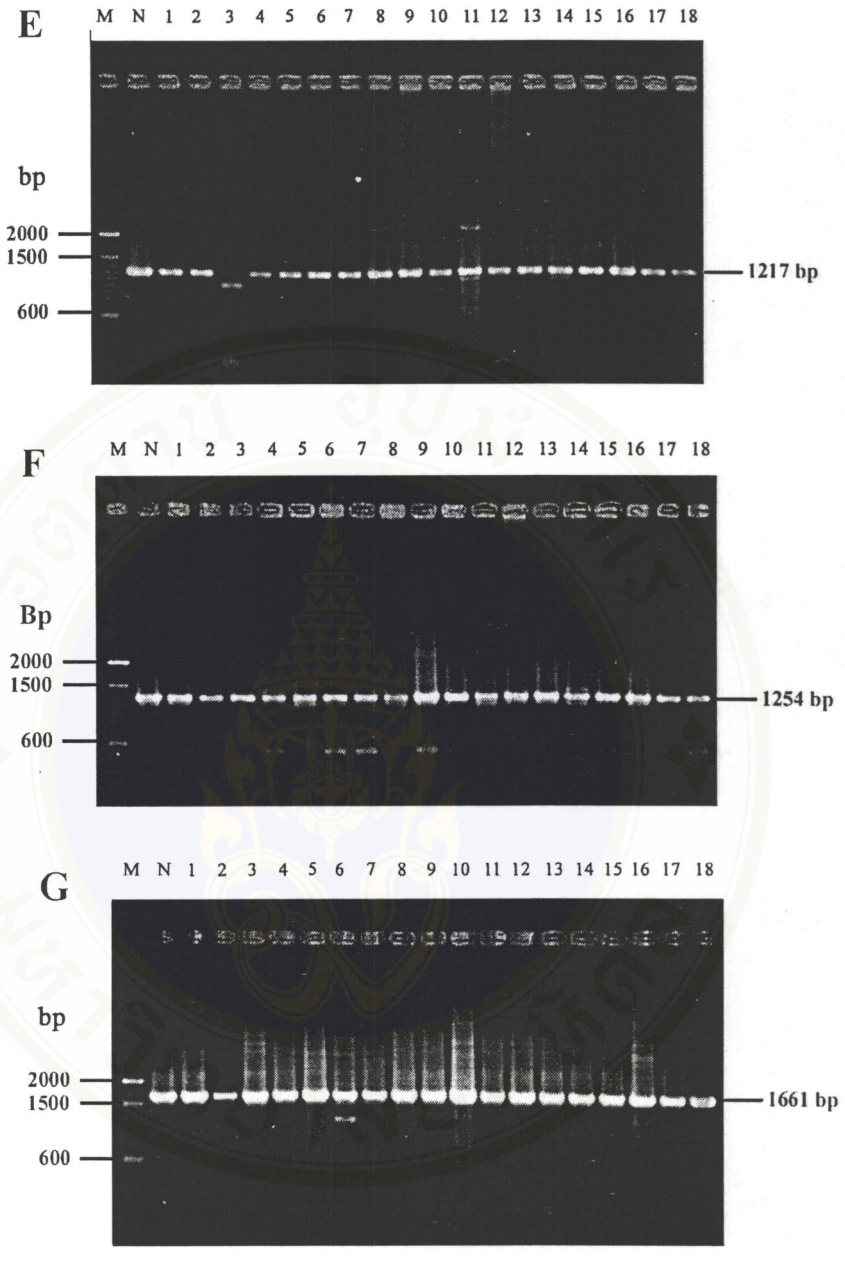


Figure 22. Nested PCR amplifications of the 7 overlapping fragments of factor VIII cDNA.

PCR products resulted from nested amplifications of factor VIII cDNA of a normal person and eighteen hemophilia A patients to obtain seven overlapping fragments (A-G), detected by 1% agarose gel electrophoresis in 1x TAE buffer. Lane M is 100 bp DNA markers. Lane N is PCR product of normal factor VIII cDNA. Lanes 1-18 are PCR products of hemophilia A patients' factor VIII cDNA. In figure 22-E, one sample (lane 3) showed a shorter size of A5 fragment.

3. Characterization of the shorter A5 fragment

To characterize the shorter A5 fragment of the patient 8/3, the normal and the shorter A5 fragments were digested with the following restriction enzymes: *Ava*II, *Hae*III, *Pst*I, and *Sau*3AI in order to locate the position of possible deletion (section 9 of the Methods). The cleavage sites of these enzymes were located on the A5 fragment by searching with the MacVector 4.5.1 computer program. In normal A5 fragment, there were one *Ava*II, three *Hae*III, two *Pst*I, and four *Sau*3AI restriction sites. The results of these restriction endonuclease analyzes are shown in Figures 23 and 24, and summarized in Table 5.

The result revealed an approximately 200 bp deletion in the shorter A5 fragment of the patient 8/3. The deletion occurred between the first cutting site of *Pst*I (about 200 bp from the 5' end) and the second cutting site of *Sau*3AI (about 470 bp from the 5' end). This deletion led to the absence of the first and the second cutting site of *Hae*III, which locate about 400 bp from the 5' end. The deletion seemed to occur within exons 15 and 16 of the factor VIII mRNA. The shorter A5 fragment was amplified by nested PCR using a pair of primers 5A.1/5B.1 to generate a 442 bp product, spanning from exon 14 to exon 16). When the 442 bp amplified product was sequenced by using the primer 5A.1 as the sequencing primer, it was found that there was a deletion of 201 bp, including the whole exon 15 (154 bp) and a part of exon 16 (47 bp) (Figure 25). This resulted in in-frame deletion of 67 amino acids in the factor VIII protein.

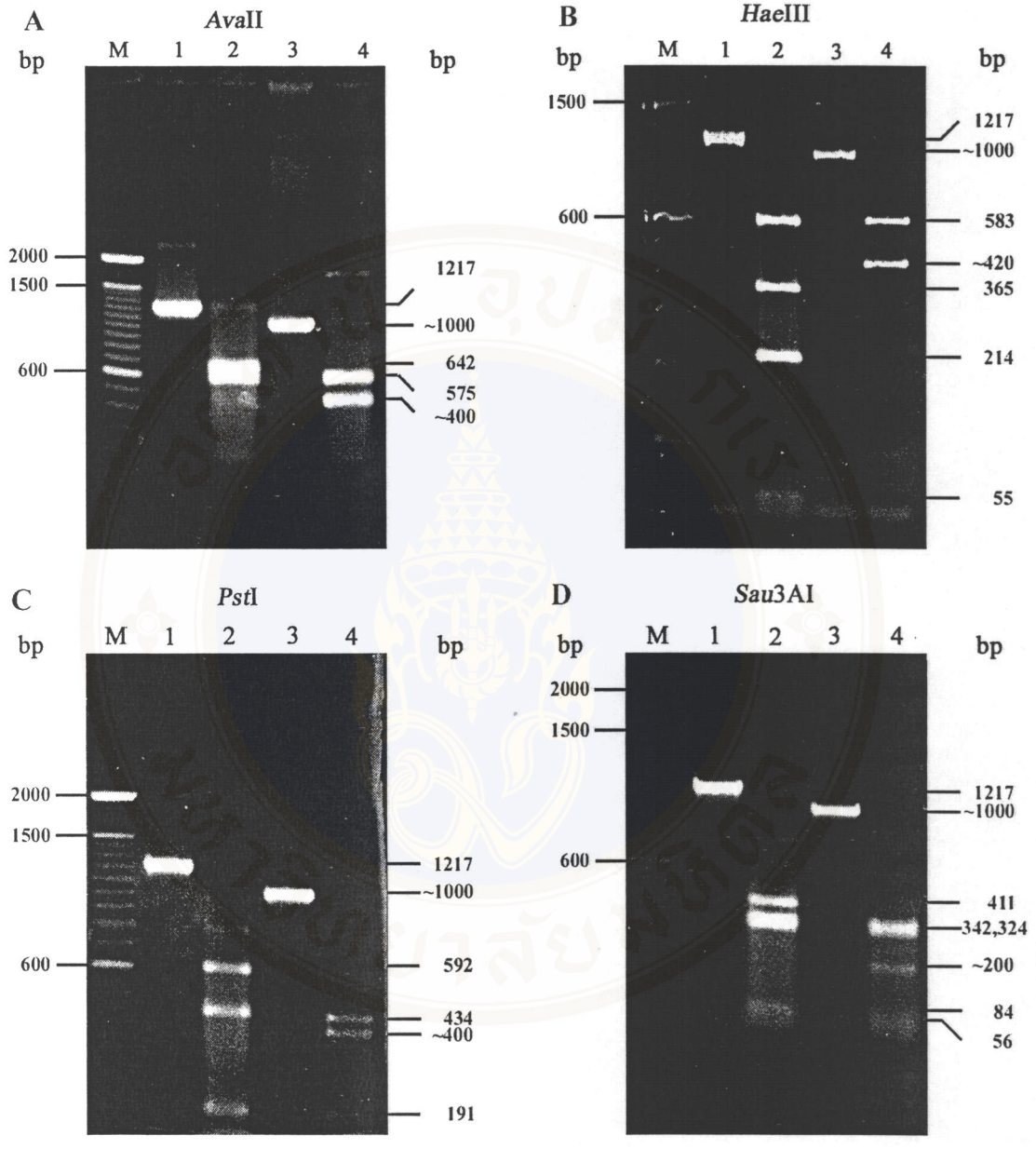


Figure 23. Restriction endonuclease analyses of the normal and shorter A5 fragments.

The A5 fragments of normal individual and the patient 8/3 who was suspected to have DNA deletion were digested with *Ava*II (A), *Hae*III (B), *Pst*I (C), and *Sau*3AI (D). Lane M is 100 bp DNA markers. Lane 1 is undigested normal A5 fragment. Lane 2 is digested normal A5 fragment. Lane 3 is undigested patient 8/3's A5 fragment. Lane 4 is digested patient 8/3's A5 fragment (see also the restriction maps in Figure 24).

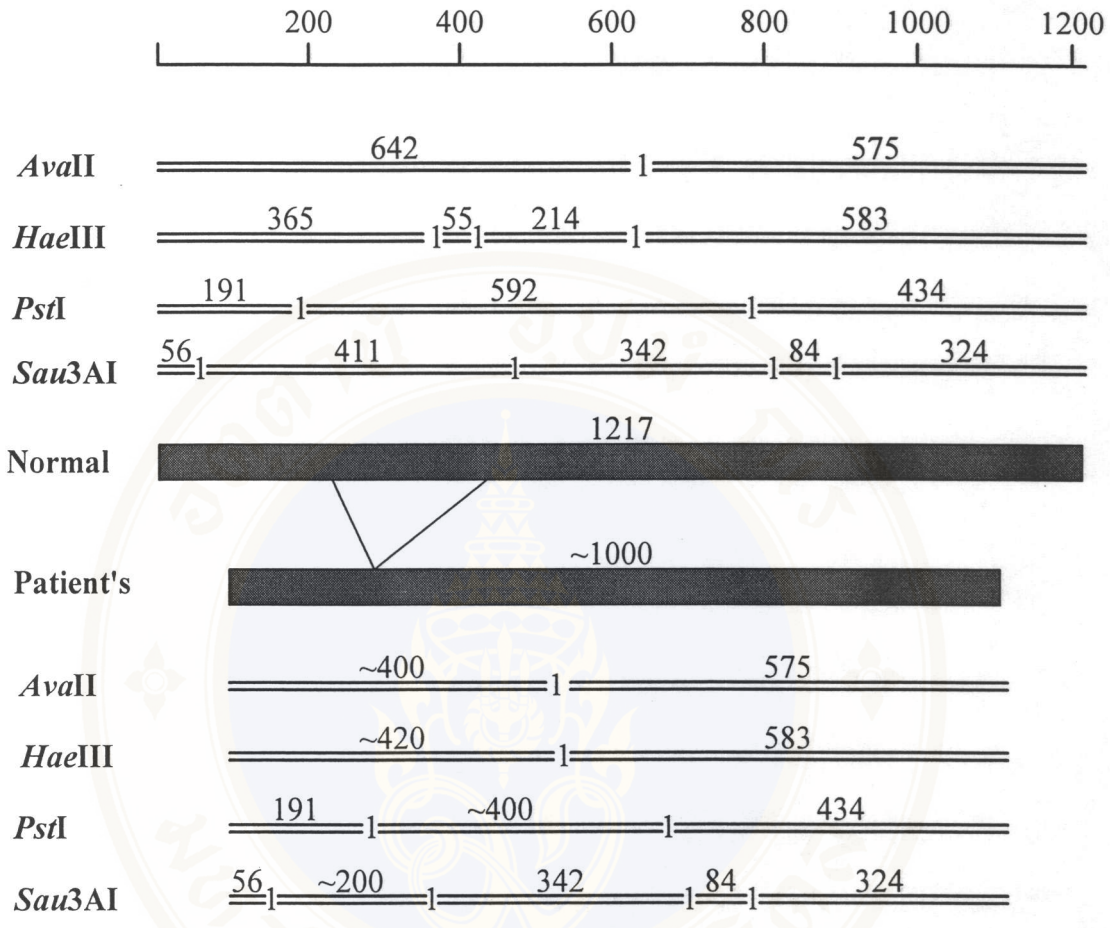
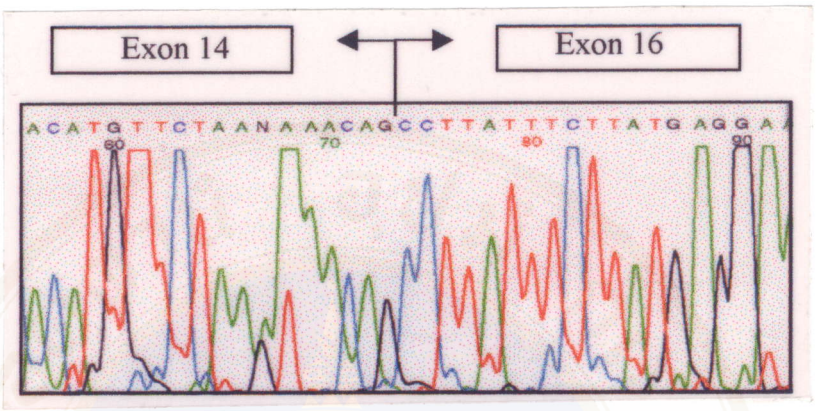


Figure 24. Restriction endonuclease maps of *Ava*II, *Hae*III, *Pst*I, and *Sau*3AI on the normal and shorter A5 fragments.

The long and short solid bars in the middle of the figure represent the normal and shorter A5 fragments, respectively. The maps of restriction enzymes on the normal and shorter A5 fragments are illustrated above and below the fragments. The sizes of digested fragments (in bp) are indicated by numbers. The shorter A5 fragment was found to have a deletion of approximately 200 bp in the region between the first cutting site of *Pst*I and the second cutting site of *Sau*3AI. This also led to disappearing of the first and second cutting sites of *Hae*III.

Table 5. Summary of restriction endonuclease analyses of the normal and the shorter A5 fragments.

Restriction enzyme	Normal PCR product (bp)	Patient's PCR product (bp)
Undigested	1,217	~1000
*Ava*II	642 575	~400 575
*Hae*III	583 365 214 55	583 ~420
*Pst*I	592 434 191	~400 434 191
*Sau*3AI	411 342 324 84 56	~200 342 324 84 56



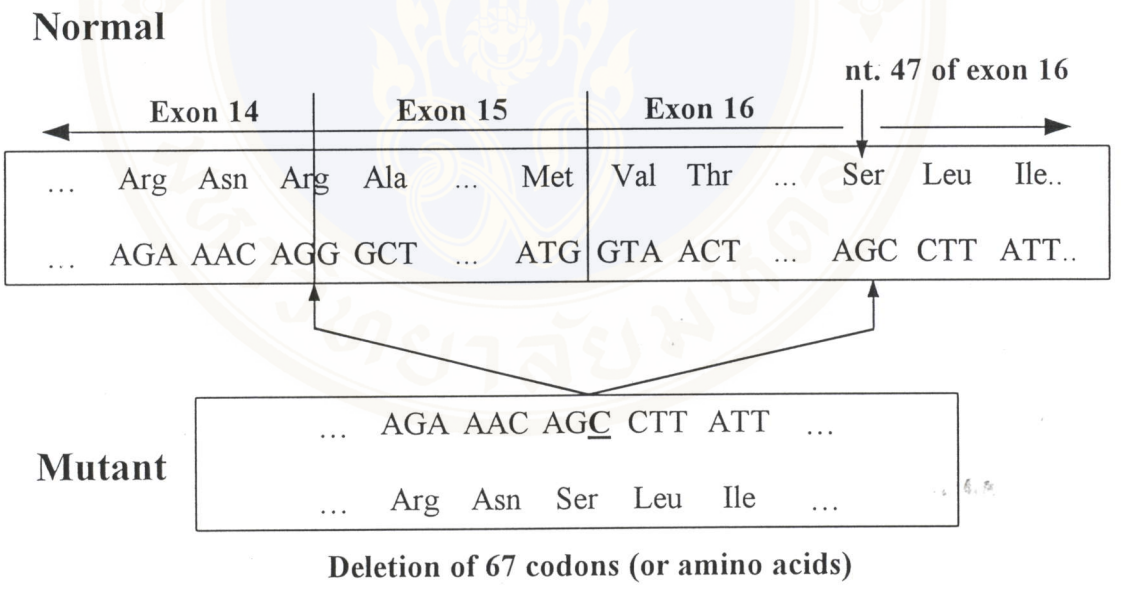


Figure 25. Sequence analysis of the patient 8/3's PCR product amplified with 5A.1 and 5B.1 primers.

The figure illustrates the chromatogram of DNA sequencing of the patient 8/3's fragment using 5A.1 primer. The result revealed a deletion of 201 bp coving the whole exon 15 (154 bp) and the first 47 bp of exon 16. This deletion resulted in deletion of 67 amino acids in the factor VIII protein.

To identify an exact cause of the deletion in the patient's factor VIII mRNA, amplifications of the patient's genomic DNA within exon 15 and exon 16 were performed by using intronic primers, F8Ex15F1/F8Ex15B1 and F8Ex16F1/F8Ex16B1, respectively (Figure 26). The amplified products did not show a large deletion within these two exons (data not shown). Therefore, it was most likely that the deletion should result from a point mutation or small deletion/insertion within or outside the coding region. The above PCR products amplified by using F8Ex15F1/F8Ex15B1 and F8Ex16F1/F8Ex16B1 primers were subsequently sequenced by using F8Ex15F1 and F8Ex16F1 as sequencing primers, respectively. Genomic sequencing of exon 15, exon 16, and the surrounding intronic regions demonstrated a transversion (GT→TT) in the donor splice site of intron 15 without a genomic deletion or mutation in other regions (Figure 27). This mutation was confirmed by sequencing of the opposite strand of DNA from the same patient, and the mutation was also detected by DNA sequencing in his brother who was also a hemophiliac (Figure 27).

Figure 26. Locations of F8Ex15F1/F8Ex15B1 and F8Ex16F1/F8Ex16B1 primers in the flanking introns of exons 15 and 16.

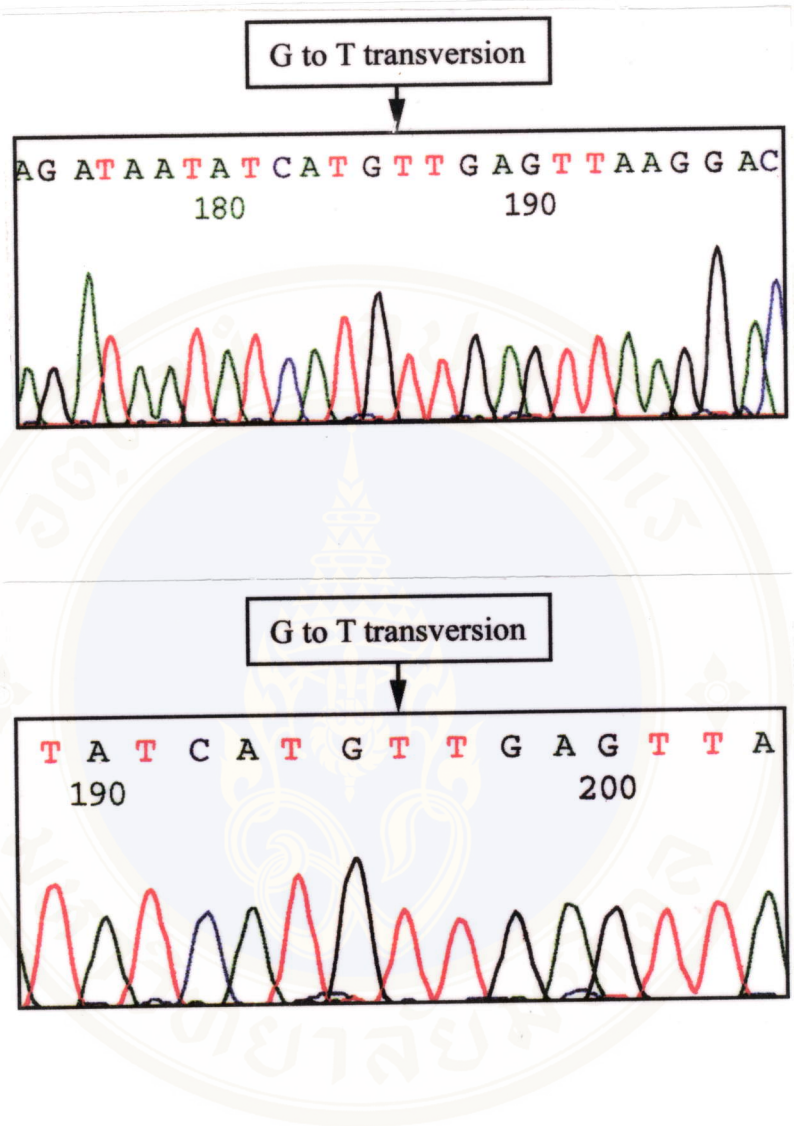


Figure 27. Sequence analyses of amplified genomic DNAs in the region of exon 15 and intron 15 from the patient 8/3 and his brother (patient 8/5).

The figure illustrates the chromatogram of genomic DNA sequencing of exon 15 and its intronic sequence from the patients 8/3 and 8/5. The results revealed the G to T transversion at the donor splice site of intron 15 in both patients.

4. Screening of mutation by single strand conformation polymorphism (SSCP)

In this study point mutations or small molecular defects in the factor VIII coding sequence of 8 hemophilia A patients were screened by SSCP (section 10 of the Methods). To simplify the analysis of the coding region, the amplification of large PCR fragments and endonuclease digestions were required before SSCP analysis. Six fragments designated as A1/1, A2/1, A3, A4, A5/1, and A6/1 fragments were amplified by using F8N1C/F8N1D, F8N2C/F8N2D, F8N3A/F8N3B, F8N4A/F8N4B, F8N5C/F8N5D, and F8N6C/F8N6D primers (section 5.2 of the Methods) (Figure 28). These products were purified (section 8 of the Methods) and subsequently digested with the restriction enzymes (section 9 of the Methods) as listed in Table 6 in order to obtain appropriate sizes of the fragments for the SSCP analysis. The electrophoresis was performed at room temperature on 10% non-denaturing polyacrylamide gel in the presence of 5% glycerol. The PCR-SSCP screening of the digested fragments showed an abnormal single-stranded DNA (458 nt) of the *Eco*RI/*Hin*dIII digested A1/1 fragment in two patients, 14/4 and 14/5, from the same family (Figure 29).

To identify mutation in these patients by DNA sequencing, a DNA segment of 812 bp was amplified by using nested PCR primer pair (Ex4L/F8N1D) and sequenced by using F8N1D as a sequencing primer. The result showed that a single base substitution (G→A) occurred on antisense-strand, which corresponded to a C→T on the sense-strand. This substitution changed codon 233 from ACA, the code for threonine, to ATA, the code for isoleucine (Figure 30).

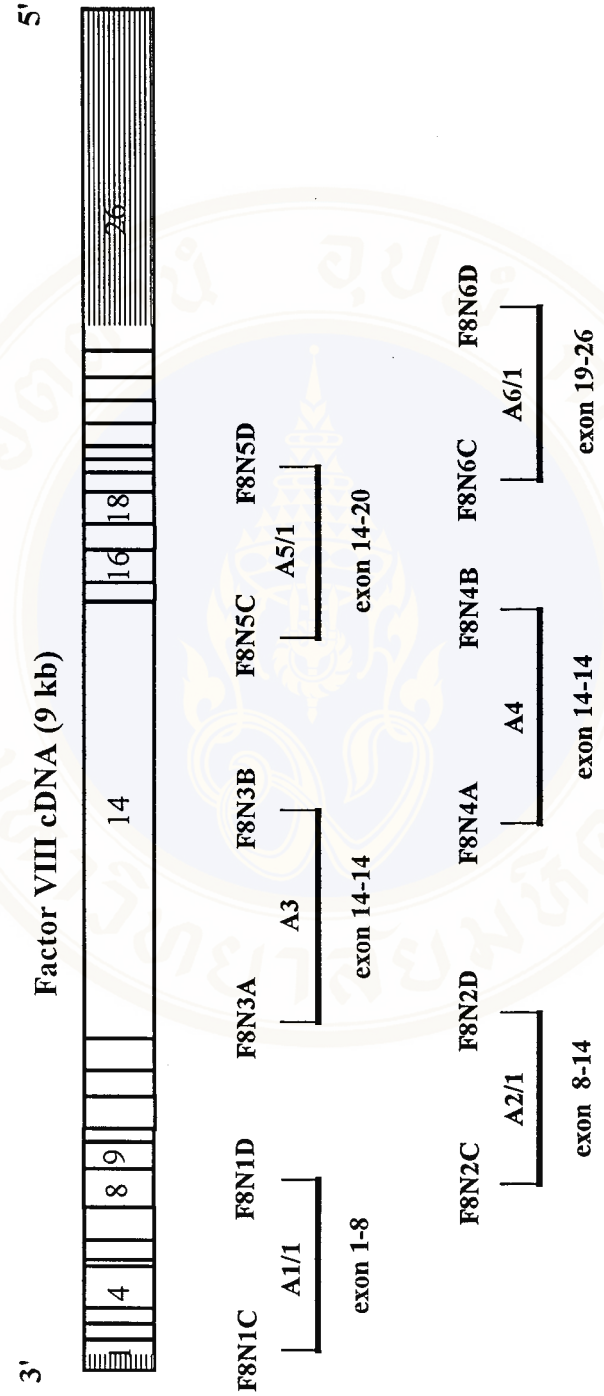


Figure 28. Locations of primers used in SSCP analysis.

Table 6. List of PCR products and restriction enzymes used for digestions prior to the SSCP analysis.

PCR primer	Fragment name	Size (bp)	Enzyme used	Size of digested fragment (bp)
F8NIC/F8N1D	A1/1	1273	*Eco*RI/*Hin*dIII	265,163,184,458,203
F8N2C/F8N2D	A2/1	1169	*Bam*HI/*Bst*NI	458,298,242,171
F8N3A/F8N3B	A3	1579	*Eco*RI/*Pst*I	176,369,1034
F8N4A/F8N4B	A4	1683	*Sca*I/*Hae*III	350,457,615
		1683	*Xcm*I	865,389,429
F8N5C/F8N5D	A5/1	1139	*Sau*96I	317,279,543
		1139	*Dra*I/*Hinc*II	562,213,364
F8N6C/F8N6D	A6/1	1073	*Dde*I	283,366,72,221,41,47,43
		1073	*Dde*I/*Xba*I	449,376,198

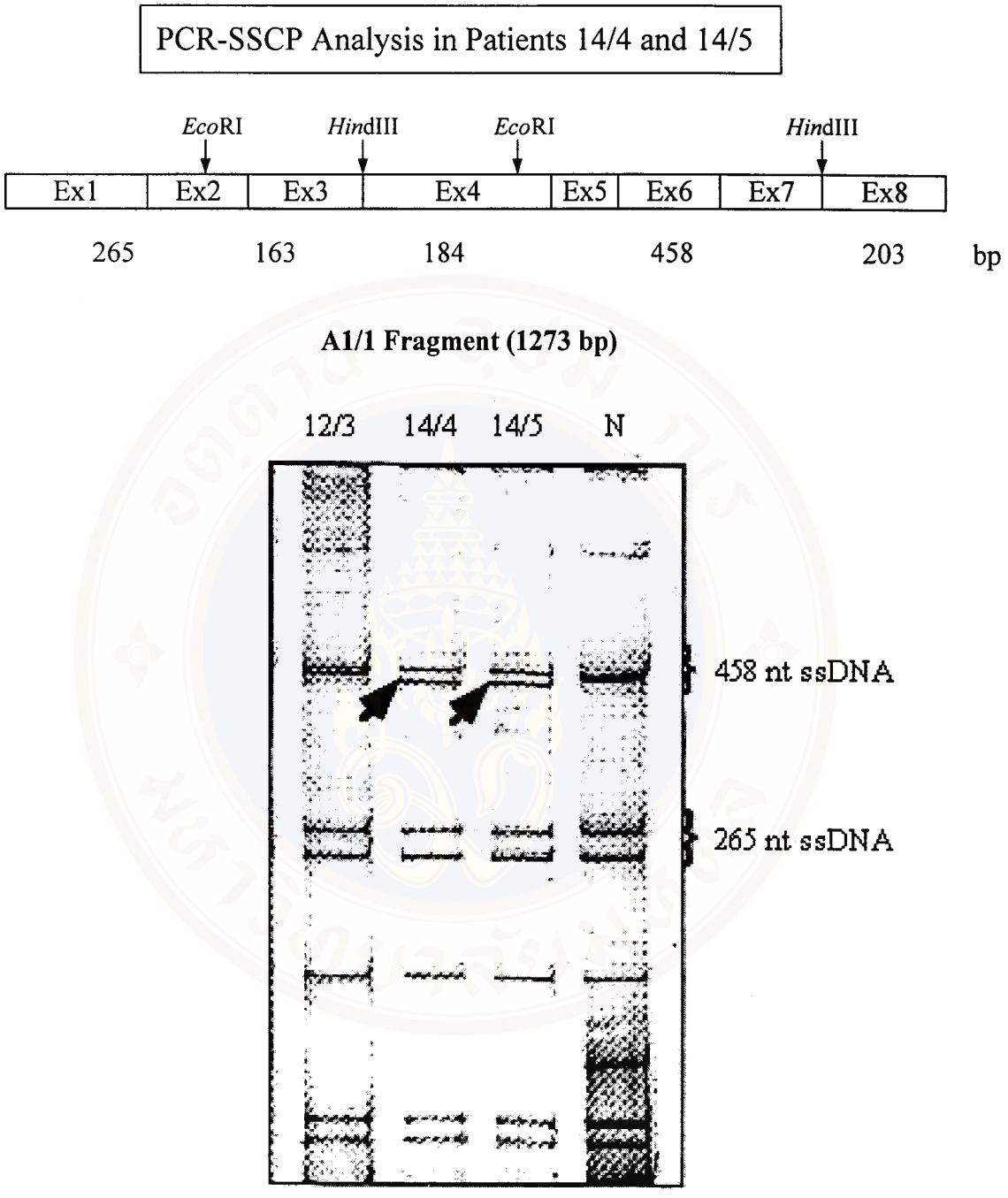


Figure 29. SSCP analysis of A1/1 fragment of patients 14/4 and 14/5, digested with *Eco*RI/*Hin*dIII.

The PCR fragment-A1/1, 1273 bp, extending from exon 1 to 8 is shown by line diagram. The digested PCR product was electrophoresed in a 10% polyacrylamide gel containing 5% glycerol at 20 mA, room temperature for 5.30 hr. Lane M shows normal mobility, lanes 2 and 3 (patients 14/4 and 14/5) show abnormal mobility in a single-stranded 458 nt fragment (arrows), and N is a normal control.

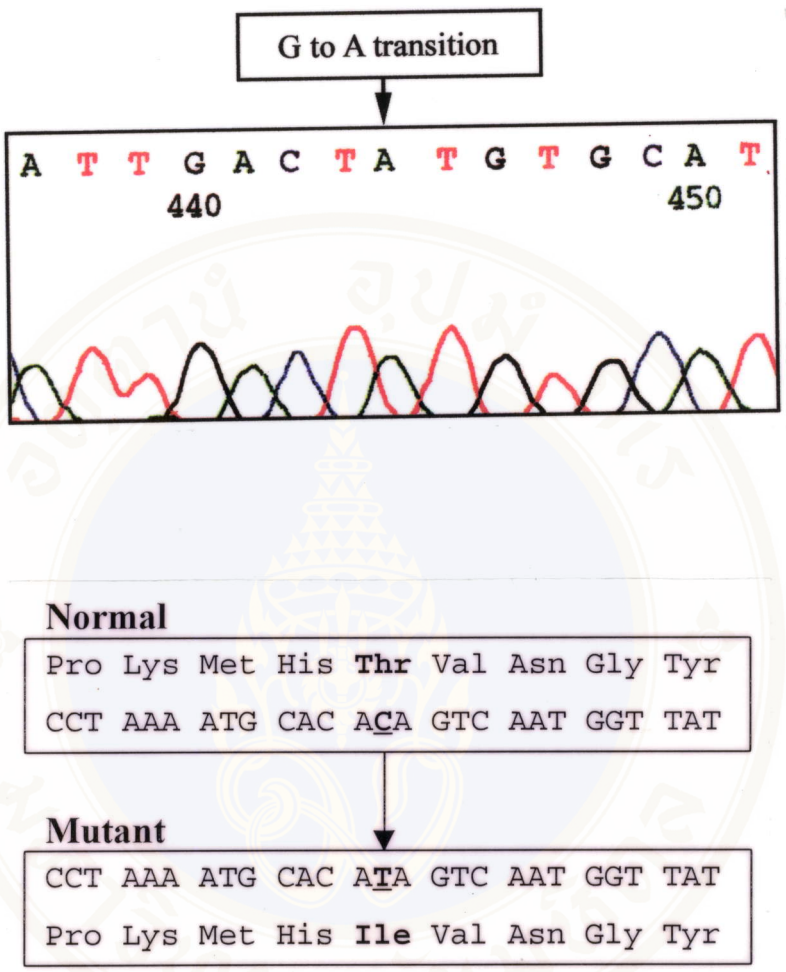


Figure 30. Automated sequencing of nested PCR product of patient 14/4's fragment-A1/1 amplified with the Ex4L and F8N1D primers.

The figure illustrates the chromatogram of cDNA sequencing of antisense-strand of patient 14/4's exon 6. The G to A substitution (indicated by an arrow) corresponded to the C to T substitution at codon 233 of exon 6. Sequences of the sense strands of normal and patient's exon 6 with the translated amino acids were compared as shown in the boxes below the figure. The mutated codon is indicated in bold.

5. Direct mutation analysis by allele-specific amplification (ASA) method in families 8 and 14

The mutations of the factor VIII gene in families 8 and 14 were analysed by the ASA technique to confirm the identified mutations in the affected persons and examine carrier status in female members. In the family 8, the PCR product from each individual was amplified in two separated reactions, with either a pair of wild type (F8Ex15F1/ASAEx15Wt) or mutant (F8Ex15F1/ASAEx15Mt) primers (Table 4.3). As the sample genotype would be inferred from the presence or absence of the product, internal control primers (F8-3'L3/F8-3'R3) were included in both reactions to ensure the success of PCR. For the family 14, another set of wild type (F8Ex6F1/ASAEx6Wt) or mutant (F8Ex6F1/ASAEx6Mt) primers were used to detect the mutation with another pair of internal control (F8Ex14L/F8Ex14R) primers. The PCR mixture for ASA was similar to that of nested PCR (section 5.2 of the Methods) with a little modification of using 6-fold lower amount of internal control primers than that of the ASA primers for the mutation analysis in the family 8. The PCR reactions in both families were performed for 40 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 35 s. The internal control products with the size of 392 and 242 bp had been shown in both normal and patient DNA samples in families 8 and 14, respectively. The amplified DNA of normal samples could be observed (241 bp for the family 8 and 159 bp for the family 14) only in the reaction containing with the wild type primers. The same size of the PCR products were produced from the patient's samples in the reaction containing the mutant primers. For patient's mother or female relatives, who were carriers, the PCR products were obtained from both wild type and mutant reactions (Figure 31).

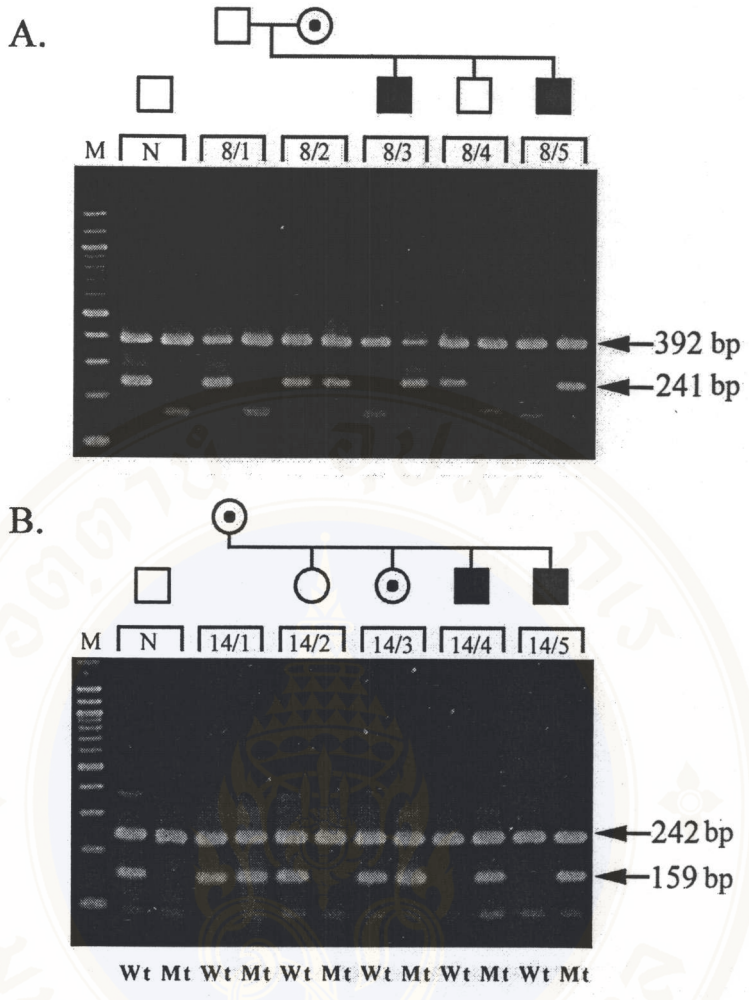


Figure 31. Detection of mutations in families 8 and 14 by ASA method.

Detections of nucleotide substitutions at donor splice site in intron15 (G or T) in the family 8, and nucleotide substitution at position 755 in exon 6 (C or T) in the family 14 using allele specific amplification (ASA) technique and gel electrophoresis. The pedigrees of both families are shown above the gels. Open square = normal male; black square = affected male; open circle = normal female; and dotted circle = carrier female. A pair of internal primers (F8-3'L3/F8-3'R3 for the family 8 and F8Ex14L/F8Ex14R for the family 14) for amplifications of 392 bp and 242 bp fragments were also added in the reactions of the families 8 and 14, respectively. (A) DNA samples of normal family members (8/1 and 8/4) showed only the product of the wild type allele (241 bp) and that of the affected members (8/3 and 8/5) showed the product of the mutant allele (241 bp). In contrast, for a member who is a carrier (8/2), the products of both alleles had been seen. (B) DNA samples of carrier members (14/1 and 14/3) showed the product size of 159 bp in both wild type and mutant reactions whereas that of normal member (14/2) and affected members (14/4 and 14/5) showed the product size of 159 bp only in a reaction corresponding to the wild type and mutant allele, respectively.

CHAPTER VI

DISCUSSION

This study was carried out as a part of a large collaborative hemophilia research project, with the aims to develope and apply the method for detection of mutations in the factor VIII gene by analyses of its mRNA and genomic DNA sequences. The strategy adopted in this study involved the isolation of the entire coding sequence of the factor VIII gene from its mRNA transcripts ectopically expressed in lymphocytes by means of long reverse transcription and polymerase chain reaction (long RT-PCR). The sequence of exon 14, the largest exon, could also be isolated from the amplification of genomic DNA. The shorter overlapping fragments of the factor VIII cDNA were generated by nested PCR for further analyses. The advantages of analysis the factor VIII mRNA are that it could reduce time and process required for the analyses, and structural mRNA abnormality could directly be detected, irrespective of the position of the causative mutation in genomic DNA.

Since the factor VIII gene inversion has been found to contribute to the molecular defects of hemophilia A in about half of the severe cases in Caucasians (38, 50), the method for analysis of the factor VIII gene inversion was also developed. Previously, Naylor et al. has demonstrated the inability to amplify across the exons 22-23 boundary by RT-PCR (42), suggesting that the detection of the factor VIII gene inversion is possible through its mRNA analysis. The RT-PCR and nested PCR technique was therefore invented and tested in this study. Ten RNA samples from severe hemphilia A patients with the factor VIII gene inversion as previously

examined by Southern blot analysis were used for re-investigation by the RT-PCR and nested PCR technique. It was found that the cDNA samples from all these 10 patients could not be amplified across the inversion breakpoint, indicating the reliability of the technique developed for detection of the factor VIII gene inversion. One RNA sample from a severe hemophilia A patient, who had never been examined by Southern blot analysis, was also investigated by the RT-PCR and nested PCR technique. The result indicated that this patient carried the factor VIII gene inversion. This was also supported by the finding that the PCR product of the fragment A6 could not be amplified by using F8N6A/F8N6B primers. Thus, this patient was unlikely to carry a point mutation at a binding site of primers. However, the possibility of a partial deletion in the region of exons 21-25 of the factor VIII gene could not be excluded.

The advantage of this RT-PCR and nested PCR technique for detection of the factor VIII gene inversion is that it is not as complicated as the Southern blot hybridization. Therefore, it can be used as the method for screening the factor VIII gene inversion in the cases with severe hemophilia A before the analysis by the Southern blot hybridization or for excluding the inversion in the cases with severe hemophilia A seclected for further mutation analysis.

The analysis of RNA samples from 18 hemophilia A patients by amplifications of 7 overlapping fragments with the long RT-PCR and nested PCR demonstrated that the patient 8/3 had the shorter A5 fragment with the size of ~1,000 bp instead of normal 1,217 bp. Restriction endonuclease mapping using the four enzymes including *Ava*II, *Hae*III, *Pst*I, and *Sau*3AI, indicated that there was a deletion of ~200 bp in the region of exons 15 and 16. DNA sequencing analysis revealed the deletion of 201 bp, including the whole exon 15 (154 bp) and a part of exon 16 (47 bp) in the A5 fragment

of the patient 8/3. This deletion in the factor VIII cDNA derived from its mRNA might occur from an intragenic deletion of the factor VIII gene or a point mutation at (or near) one of the splice signals of introns 14 and 15. Genomic amplifications and sequencing analyses of exons 15 and 16 and their surrounding intronic regions showed that the mutation did not result from an intragenic deletion. However, this demonstrated that a mutation at the donor splice site of intron 15, IVS15+1G>T, occurred in the patient 8/3. The G to T transversion changed the first nucleotide in donor splice-site consensus sequence of intron 15 from GT to TT.

Although this mutation had previously been described (94), its effect on the RNA processing were not studied. The result of cDNA (or mRNA) analysis in the present study demonstrated that this mutation not only inactivated the donor splice site of intron 15 but also activated a cryptic splice site at the nucleotides 46^{th}-47^{th} within exon 16, resulting in a complete skipping of exon 15 and a partial deletion of exon 16. It was found from examination of the nucleotide sequence of exon 16 that it exhibits the characteristics of an internal cryptic splice site, a polypyrimidine-rich tract (C/T) preceeding AG dinucleotide (Figure 32). This cryptic splice site seems to be sensitive to activation by mutations in the surrounding regions. The activation of this cryptic splice site has been observed in a case with severe hemophilia A due to a missense (R1781H) mutation in exon 16 of the factor VIII gene, which also resulted in the deletion of the first 47 nucleotide of exon 16 in approximately 80% of the processed factor VIII mRNA (95).

The amount of PCR product of the shorter A5 fragment from the patient 8/3 was less than that of the normal A5 fragment from the normal individual, indicating the instability of abnormal mRNA. The deletion might also lead to a partial degradation

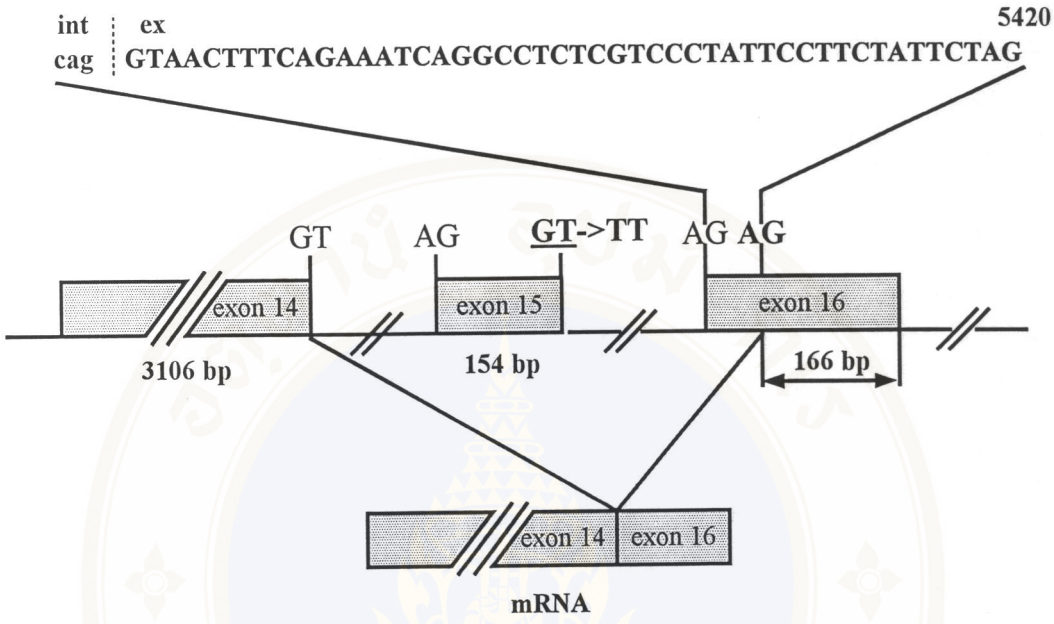


Figure 32. Schematic representation of mutation at the donor splice site in intron 15 causing splicing defect of the factor VIII mRNA with exon 15 skipping and 47-nt deletion of exon 16 in the patient 8/3.

The coding regions are indicated by shaded boxes. Exonic (ex) and intronic (int) sequences are given in uppercase and lowercase letters, respectively. Normal donor (GT) and acceptor (AG) splice sites of introns 14 and 15, the nucleotide substitution (GT>TT) at the donor splice site of intron 15, and a cryptic acceptor splice site (AG) within exon 16 are indicated. This splicing defect resulted in a deletion of exon 15 (154 bp) and a part of exon 16 (47 bp).

of the defective mRNA. The deletion of 201 nucleotides in the factor VIII mRNA would result in the in-frame deletion of 67 amino acids, between residues 1722-1788, in the A3 domain of the factor VIII protein, which is responsible for binding to the light chain of factor IXa and APC. Since it was apparent that there was no normally spliced factor VIII mRNA from the patient 8/3, the normal factor VIII protein should not be produced. The observation that the patient 8/3 had an undetectable level of the factor VIII procoagulant activity (FVIII:C <1%) indicated that the abnormal factor VIII protein with the intramolecular truncation of 67 amino-acid residues was also not stable.

Eight cDNA samples from the patients who did not have the factor VIII gene inversion or RNA processing defect were screened for mutation by the SSCP analysis (71, 72). The use of SSCP to detect point mutations in the genes is now widely applied because of its simplicity and versatility (96-100). The sensitivity of SSCP analysis in a single run is generally believed to be about 80% if fragments are shorter than 300 bp (101, 102), although it is variable depending on both target sequence and experimental conditions (103). The sequences of different amplified regions have been shown to affect the sensitivity of SSCP analysis (103). Many experimental parameters have been empirically found to affect the sensitivity of SSCP analysis. The electrophoretic mobility of the single-stranded DNA under non-denaturing condition can be influenced by electrophoretic conditions; for example, acrylamide cross linking, pore size of the polyacrylamide gel, temperature, size of DNA fragment, and presence of additives such as glycerol.

The ratio of acrylamide cross linking used in the SSCP protocols is very variable. Many groups have shown that gels with higher percentage of acrylamide and

lower cross-linking (i.e. low ratio of *N,N'*-methylene *bis*-acrylamide to acrylamide) can detect more mutations (86, 103, 104). Orita *et al.* (72) used a polyacrylamide gel of 5 or 6% for the SSCP analysis, but Teschauer *et al.* (105) found that clear result was obtained when the gel with a high acrylamide concentration was used. Two acrylamide concentrations, 10 and 12%, have been tested and the results showed no difference between these two concentrations. Therefore, 10% polyacrylamide gel with a low cross-linking (49:1) was chosen for the SSCP analysis of the factor VIII gene mutations in this study.

Many SSCP protocols recommended the addition of glycerol to the gel matrix. The addition of 5% or 10% glycerol in gel, buffered with Tris-borate-EDTA (TBE) is generally recognized to enhance separation of different mutant fragments and to improve overall sensitivity, but the reason for this glycerol effect has not been understood (86). However, Kukita *et al.* (106) found that glycerol causes a reduction of pH in the buffer by the reaction of glycerol and borate ion. The glycerol affects mobility of single-stranded DNA only when the borate ion is present in the buffer. In this study the SSCP analysis was performed in TBE buffer with 10% polyacrylamide gel in the presence of 5% glycerol, by running at room temperature.

In this study, a similar mobility shift indicating a mutation of the factor VIII gene was found in 2 of 8 patients who were sibs in the same family. Absence of mobility shifts in the remaining six samples could be due to several reasons. Firstly, the mobility shifts could not be observed due to large sizes of DNA fragments. Although the nested PCR products of the factor VIII cDNA were digested into smaller fragments with combinations of restriction enzymes before analysis by SSCP, many fragments were still larger than 300 bp, the recommended maximal size for SSCP.

Secondly, the mutations might locate at the terminal 5' or 3' region of the DNA fragments, which would not have any effect on the conformations of single stranded DNAs. Thirdly, it might be possible that resolution of the SSCP gel was not optimal. The gel used in the present study was thicker and its length was shorter than the conventional radioactive SSCP gel, since it was convenient for silver staining and the use of radioisotope was avoided. In addition, the mutations might locate in the controlling regions of the factor VIII gene expression, further upstream or downstream to the regions analyzed, or in the intron regions which might not interfere with RNA processing but resulted in low factor VIII mRNA level.

The SSCP analysis of digested PCR product of fragment A1/1 from the patient 14/4 and 14/5 showed the mobility shift of a single stranded DNA (Figure29). The DNA sequencing of nested PCR product from the patient 14/4 demonstrated the C to T substitution at codon 233, changing this codon from ACA to ATA, in exon 6. Thus, the amino acid at the position 233 of factor VIII was altered from threonine to isoleucine (T233I) (Figure30). This mutation has never previously been reported. The amino acid residue 233 is in the A1 domain in the heavy chain of the factor VIII protein. In preactivated factor VIII, the A1 and A2 domains are connected in the heavy chain. When it is activated, the heavy chain is cleaved by thrombin or factor Xa and the two domains are disconnected. The A1 domain binds to the A2 and A3 domains in the activated factor VIII.

Threonine at the position 233 is highly conserved among all factor VIII and factor V; it is a serine (S), which is also an uncharge polar amino acid, at this position in human, mouse, and rat ceruloplasmin (Figure 33). Other four residues next to

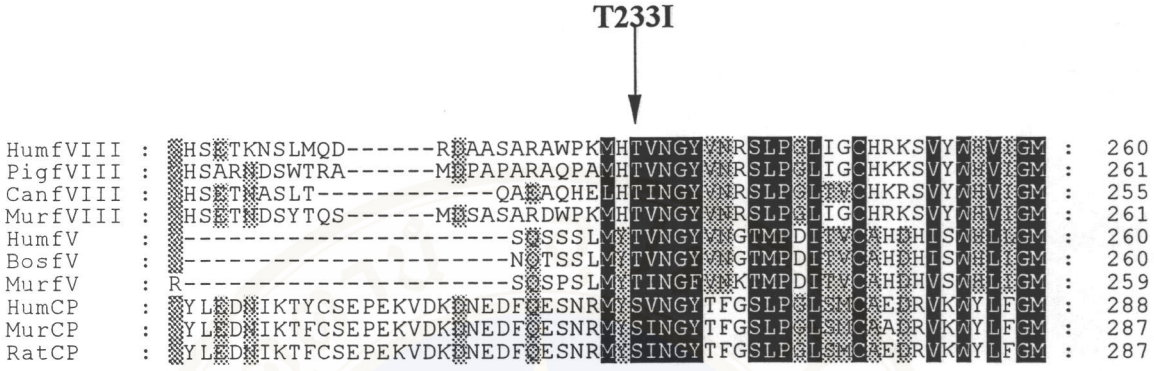


Figure 33. Partial amino acid sequence lineups of human factor VIII with other homologous proteins.

The figure shows the amino acid sequence alignment between human factor VIII and other homologous proteins. The highly conserved sequences are indicated in black areas. The arrow indicates position of threonine 233.

HumfVIII	=	human factor VIII,	accession no. M14113.
PigfVIII	=	pig factor VIII,	accession no. U49517.
CanfVIII	=	canine factor VIII,	accession no. AF049489.
MurfVIII	=	mouse factor VIII,	accession no. L05573.
HumfV	=	human factor V,	accession no. M16967.
BosfV	=	bovine factor V,	accession no. M81441.
MurfV	=	mouse factor V,	accession no. U52925.
HumCP	=	human ceruloplasmin,	accession no. M13699.
MurCP	=	mouse ceruloplasmin,	accession no. U49430.
RatCP	=	rat ceruloplasmin,	accession no. L33869.

threonine are also highly conserved, forming a motif of 'TVNGY' at the positions 233-237. The substitution of valine (V) at the residue 234 by phenylalanine (F), has previously been reported to also result in hemophilia A (107). This suggests the structural or functional significance of the highly conserved 'TVNGY' motif. Theonine at the position 233, which is an uncharge polar amino acid, may involve in interaction with other residues to maintain protein structure and stability. The substitution of threonine by isoleucine which is a non-polar amino acid may result in a structural change. It is not known whether this structural change will lead to the alteration in its stability or function or both stability and function. The two patients (14/4 and 14/5) in this family had severe hemophilia A with FVIII:Cs of 2.5% and 1.7%, respectively, indicating its impaired function. Since the factor VIII antigen was not assayed, its stability is unknown.

To confirm the two mutations, IVS15+1G>T in family 8 and T233I in family 14, and to determine the carrier status of female members in these two families, the method for direct mutation analysis by allele specific amplification (ASA) was developed. The ASA analysis in the family no. 8 (Figure 31A) showed that the IVS15+1G>T mutation was present in the mother (8/2) and two patients (8/3 and 8/5) but absent in the father (8/1) and the unaffected son (8/4). This results were consistent to the phenotypes in the family members and the results of sequencing analysis. Thus, the mother (8/2) was the carrier of this mutation. The ASA analysis in the family no. 14 (Figure 31B) illustrated that the T233I mutation was present in the mother (14/1), the second daughter (14/3), and two patients (14/4 and 14/5) but absent in the first daughter (14/2). Thus, the mother (14/1) and the second daughter (14/3) were carriers of the mutation but the first daughter (14/3) was not.

In conclusion, this study has demonstrated that the factor VIII mRNA illegitimately transcribed in peripheral blood lymphocytes could be used for the analyses of factor VIII gene inversion, the mutation causing splicing defect, and point mutation. The analysis of factor VIII gene inversion by RT-PCR and nested PCR from mRNA is useful for initial screening before the study by Southern blot hybridization or the examination of other mutations. The direct analysis of the factor VIII mRNA from hemophilia A patients allows the detection of mutation causing splicing defects without the requirement of a complicated *in vitro* expression study. And, the screening and characterization of point mutations by the factor VIII mRNA analysis would help to reduce time and workload required. This study has also shown that the carrier detection by direction mutation analysis using the ASA technique is possible.

CHAPTER VII

SUMMARY

1. The RT-PCR and nested PCR for screening of the factor VIII gene inversion was developed by the specific amplifications of the inversion breakpoint region located between exons 22 and 23 and the 5' region of factor VIII transcript as internal positive control. The presence of factor VIII gene inversion was indicated by the absence of amplified product from the inversion breakpoint region.

2. The factor VIII coding sequence could be isolated and amplified from its mRNA transcript by long RT-PCR and the long factor VIII cDNA was fractionated by nested PCRs into seven overlapping fragments.

3. From the screening of factor VIII cDNAs of hemophilia A patients, a mutation of the factor VIII gene causing splicing defect was identified. The mutation occurred from a nucleotide substitution at the donor splice site of intron 15 (IVS15+1G>T), resulting in the deletion of 201 nucleotides due to the skipping of 154-nt sequence of exon 15 and partial deletion of 47-nt sequence of exon 16 in the factor VIII transcript. This consequently caused the in-frame deletion of 67 amino acids, between residues 1722-1788, in the A3 domain of the factor VIII protein.

4. By the PCR-SSCP analysis, a novel missense mutation (T233I) due to the C to T substitution at codon 233 (ACA to ATA) in exon 6 was identified. The T233I amino-acid substitution occurred in a highly conserved region in the A1 domain of the factor VIII protein, predicted to affect the structure and function of the factor VIII protein.

5. The method for direct mutation analysis based on allele specific amplification (ASA) was developed to detect the two mutations identified and found to be possible for determination of carrier and non-carrier statuses in the two hemophilia A families examined.

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