SCREENING FOR POINT MUTATIONS IN THE FACTOR VIII GENE BY DENATURING GRADIENT GEL ELECTROPHORESIS

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SCREENING FOR POINT MUTATIONS IN THE FACTOR VIII GENE BY DENATURING GRADIENT GEL ELECTROPHORESIS

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"Imagination is more important than knowledge."

Albert Einstein

Emeklerinin karşılığını hiçbir zaman ödeyemeyeceğim

ZEHRA TEMEL'E

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ABSTRACT

In genetic disorders such as hemophilia A, accurate carrier identification and prenatal diagnosis is achieved by molecular approaches namely linkage analysis and direct identification of mutations.

DNA linkage analysis is the major diagnostic approach because of the large size of the FVIII gene causing hemophilia A. However, linkage analysis has a number of limitations such as the requirement of the participation of many family members and the need of an informative and preferably intragenic marker for the family. Family members of five hemophilia A afflicted families that requested carrier identification and/or prenatal diagnosis have been analyzed with three markers that had previously been used in linkage analysis of hemophilia A. In three families expecting mothers were diagnosed as non-carriers by exclusion analysis. The two prenatal diagnosis revealed that one fetus was a normal female and the other one was an affected male.

In order to improve the effectiveness of linkage analysis in families afflicted with hemophilia A, the preliminary analysis of a hypervariable $(CA)_n$ repeat polymorphism located at Intron 13 of the Factor VIII gene was carried out. Seven families were analyzed using $(CA)_n$ repeat polymorphisms. In two families carrier identification and prenatal diagnosis carried out with previous markers were confirmed with $(CA)_n$ repeat analysis.

Denaturing Gradient Gel Electrophoresis (DGGE) is one of the screening methods used to identify point mutations rapidly, in large genes. It involves the separation of DNA fragments according to their melting properties in a gel system that contains a linear gradient of DNA denaturants. Partially melted fragments are required for separation of normal and mutant DNA, where mutations fall in the melted region of the fragments. Ten per cent of the FVIII gene was screened by analyzing exons 11, 23, and 24 of 78 Turkish patients. One putative mutation in Exon 11 and two putative mutations in Exon 23 were detected. Partial sequencing of Exon 11 from the patient presumed to have a base change did not reveal any sequence alteration when compared with the sequence of Exon 11 in a normal individual. This work has initiated and established the use of DGGE analysis in screening for mutations in the factor VIII gene of Turkish patients with the aim of providing accurate prenatal diagnosis and studying the molecular biology of the gene.

ÖZET

Hemofili A gibi genetik hastalıklarda taşıyıcı belirlemesi ve doğum öncesi tanıda kesinliği sağlayan moleküler yaklaşımlar, bağlantı analizi ve mutasyonların doğrudan tespitidir.

DNA bağlantı analizi, hemofili A'ya sebep olan FVIII geninin büyüklüğü nedeniyle en önemli tanı aracıdır. Diğer taraftan, bağlantı analizi, bir çok aile bireyinin katılımını gerektirmesi, o aile için informatif ve tercihen geniçi bir markör ihtiyaç duyulması gibi bir takım sınırlamalara sahiptir. Taşıyıcı belirlemesi veya doğum öncesi tanı isteyen beş hemofili A ailesinin bireyleri, daha önce hemofili A'nın bağlantı analizinde kullanılan üç markörle incelendi. Üç ailede anne adaylarının taşıyıcı olmadığı belirlendi. İki doğum öncesi tanı, fetüslerden birinin normal ve diğerinin hasta olduğunu gösterdi.

Hemofili A ailelerinde bağlantı analizini daha etkin kılmak amacıyla FVIII geninin 13. intronunda bulunan $(CA)_n$ ardışık dizi polimorfizm analizi çalışmaları başlatıldı. Yedi aile bu polimorfizm için analiz edildi. İki ailede daha önce diğer markörlerle yapılan taşıyıcı belirlemesi ve doğum öncesi tanı sonuçları $(CA)_n$ ardışık dizi polimorfizm analizi ile doğrulandı.

Denatüre Edici Gradyent Gel elektroforezi, büyük genlerde mutasyonları tespit etmekte kullanılan hızlı tarama metodlarından biridir. Bu metod, DNA parçalarının, erime (çözülme) özelliklerine dayanarak DNA denatüre edici ajanların lineer gradyentini içeren bir gel sisteminde ayrılmalarını içerir. Kısmen çözülmüş DNA parçaları - mutasyonlar, DNA parçalarının bu çözülmüş bölgelerindedir - normal ve mutant DNA'ların ayrılması için gereklidir. FVIII geninin yüzde 10'u, 78 Türk hastasında 11., 23. ve 24. eksonları analiz etmek suretiyle tarandı. Ekson 11'de bir ve Ekson 23'de iki olası mutasyon saptandı. Ekson 11'de olası mutasyonu taşıyan hastanın kısmi dizi analizi normal bireylerin dizileriyle karşılaştırıldığında hiçbir dizi değişikliği görülmedi. Bu çalışma, FVIII geninin moleküler patolojisini araştırmak ve Türk hastalarında kesin doğum öncesi tanı olanağı sağlamak amacıyla, DGG elektroforezinin FVIII gen mutasyonlarını tarama çalışmasını başlatmıştır.

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ABBREVIATIONS

APS	ammonium peroxidisulphate
bp	base pair
BPB	bromophenol blue
BSA	bovine serum albumin
ddNTP	2', 3'- dideoxynucleoside 5'- triphosphate
DDT	DL- dithiothreitol
DGGE	denaturing gradient gel electrophoresis
DIG	digoxigenin
dNTP	2'- deoxynucleoside 5'- triphosphate
dsDNA	double-stranded DNA
EDTA	ethylenediaminetetraaceticacid
EtBr	ethidium bromide
F	factor
FVIII:Ag	factor VIII antigen
FVIII:C	factor VIII coagulant activity
h	hour
min	minute
o/n	overnight
PCR	polymerase chain reaction
PT	prothrombin time
PTT	partial thromboplastin time
RE	restriction enzyme
RFLP	restriction fragment length polymorphism
rt	room temperature
SDS	sodium dodecyl sulphate
TEMED	N, N, N', N'- tetramethylethylenediamine
TF	tissue factor
TT	thrombin time
UV	ultraviolet
XC	xylene cyanole
VNTR	variable number of tandem repeat
vWF:Ag	von Willebrand factor antigen

I. INTRODUCTION

A. Hemostasis and Blood Coagulation

Hemostasis is the term applied to the process that regulates the loss of blood from the circulatory system following injury. It involves three interrelated physiological mechanisms: constriction of blood vessels, aggregation of blood platelets to damaged subendothelial surfaces and the formation of fibrin clots (1). Abnormal function of at least one mechanism may result in excessive bleeding or hemorrhage.

The normal procedure of hemostasis begins when the vascular endothelium is damaged. Exposed subendothelial structures attract platelets and induce their loose aggregation. These components in turn initiate the generation of thrombin, which aggregates platelets irreversibly and causes the laying down of clot, a platelet-fibrin network that is an effective barrier against further escape of blood and a scaffold for repair of vessel damage. Simultaneously, limiting processes are activated that confine hemostasis to the site of injury. Finally, lysis of the platelet-fibrin network occurs when the vascular endothelium is generated (2).

The hemostatic system involves complex interactions among a set of discrete plasma proteins (Table I.1) (3). Enzyme precursors, or zymogens, are normally present in the blood, but possess essentially no biologic activity. The proteins are transformed to a trypsin-like protease (i.e., they are activated) either by a conformational change or by scission of peptide bonds via action of a converting enzyme. The rate of this reaction may be accelerated by a non-enzymatic protein cofactor, which may act either by altering zymogen conformation or by binding converting enzyme and zymogen together on a phospholipid surface.

Two different series of proteolitic reactions are present in the hemostatic system. The first is the clotting, or coagulation mechanism, the end product of which is thrombin. The second is the fibrinolytic mechanism, the end product of which is plasmin. The major role of thrombin is to initiate formation of the fibrin clot by cleaving specific peptide bonds in the plasma protein fibrinogen. Plasmin breaks down the clot by hydrolyzing different peptide bonds in the fibrin molecule.

Table I.1. The components of the blood coagulation cascade; their chromosomal locations, properties of their genes and mRNAs, their concentration and function in blood (3).

Component	Molecular Weight	Gene (kb)	Chromosome	mRNA (kb)	Exons	Plasma Concentration (μg/ml)	Function
Prothrombin	72,000	21	11p11-q12	2.1	14	100	Protease zymogen
Factor X	56,000	22	13q34	1.5	8	10	Protease zymogen
Factor IX	56,000	33	Xq26-27.3	2.8	8	5	Protease zymogen
Factor VII	50,000	13	13q34	2.4	8	0.5	Protease zymogen
Factor VIII	330,000	186	Xq28	9.0	26	0.1	Cofactor
Factor V	330,000			7.0		10	Cofactor
Factor XI	160,000	23			15	5	Protease zymogen
Factor XII	80,000	12	5	2.4	14	30	Protease zymogen
Fibrinogen	340,000					3000	Structural
Aα chain	66,500		4q26-q28		5	-	
Bβ chain	52,000		4q26-q28		8	-	
γ chain	46,500		4q26-q28		9	-	
Protein C	62,000	11		1.8	8	4	Protease zymogen
Protein S	80,000			2.4		25	Cofactor
vWF	225,000 x n[a]	175	12pter-p12	8.5	>42	10	Adhesion
Tissue factor	37,000		1pter-p12	2.1		0.0	Cofactor/initiation

[a] n denotes number of subunits, where the subunit M_r is 225,000

The generation of thrombin can be subdivided into two major sets of reactions: the activation of factor X (FX) and the subsequent conversion of prothrombin to thrombin. The activation of FX proceeds through either the intrinsic or the extrinsic coagulation cascade. The activation of prothrombin is a common pathway.

The initiation of the intrinsic pathway involves the activation FXII to FXIIa (the suffix "a" indicates that the factor is in the "activated" form), a reaction that is promoted by certain surfaces such as glass or collagen (Figure.I.1) (4). FXIIa, with high-molecular-weight-kininogen (HMWK) as cofactor, converts FXI to FXIa. FXIa converts FIX to FIXa in the presence of Ca^{2+}. FIXa, in complex with FVIIIa on membrane surfaces, catalyzes the activation of FX to FXa. FXa activates prothrombin to thrombin together with Ca^{2+} and FVa bound to membrane surfaces. Thrombin converts fibrinogen to fibrin by cleavage of two peptide bonds.

The expression of tissue factor with vessel injury initiates the extrinsic pathway by participating as a cofactor in the activation of FVII to FVIIa. The FVIIa-TF complex activates FX and FIX in the presence of Ca^{2+}. FXa activates prothrombin to thrombin and thrombin converts fibrinogen to fibrin.

FXII, FXI, FIX, FX, FVII, and prothrombin are zymogens of serine proteases. FX, FIX, FVII, and prothrombin can also be classified as vitamin-K-dependent serine proteases. They contain a γ-carboxyglutamic acid containing domain (gla domain), synthesized by a vitamin-K-dependent reaction (3). Ten to 12 of glutamic acids in the amino-terminal domain are carboxylated to form γ-carboxyglutamic acid (1). This domain possesses unique calcium-dependent membrane-binding properties. HMWK, TF, FVIII, and FV are the cofactors of the coagulation cascade.

Upon stimulation at the site of tissue injury, platelets adhere to the injured surface. This interaction requires von Willebrand's factor (vWF), which is synthesized in endothelial cells. It binds to a specific receptor on the platelet membrane. vWF acts as a carrier or a stabilizer of FVIII and they can circulate in plasma as a non-covalently linked complex.

Finally, in the coagulation cascade the conversion of soluble fibrinogen to insoluble fibrin forms a filamentous network and stabilizes the platelet plug. Consequently, the deficiency of any element in this pathway results in a prolonged clotting time.

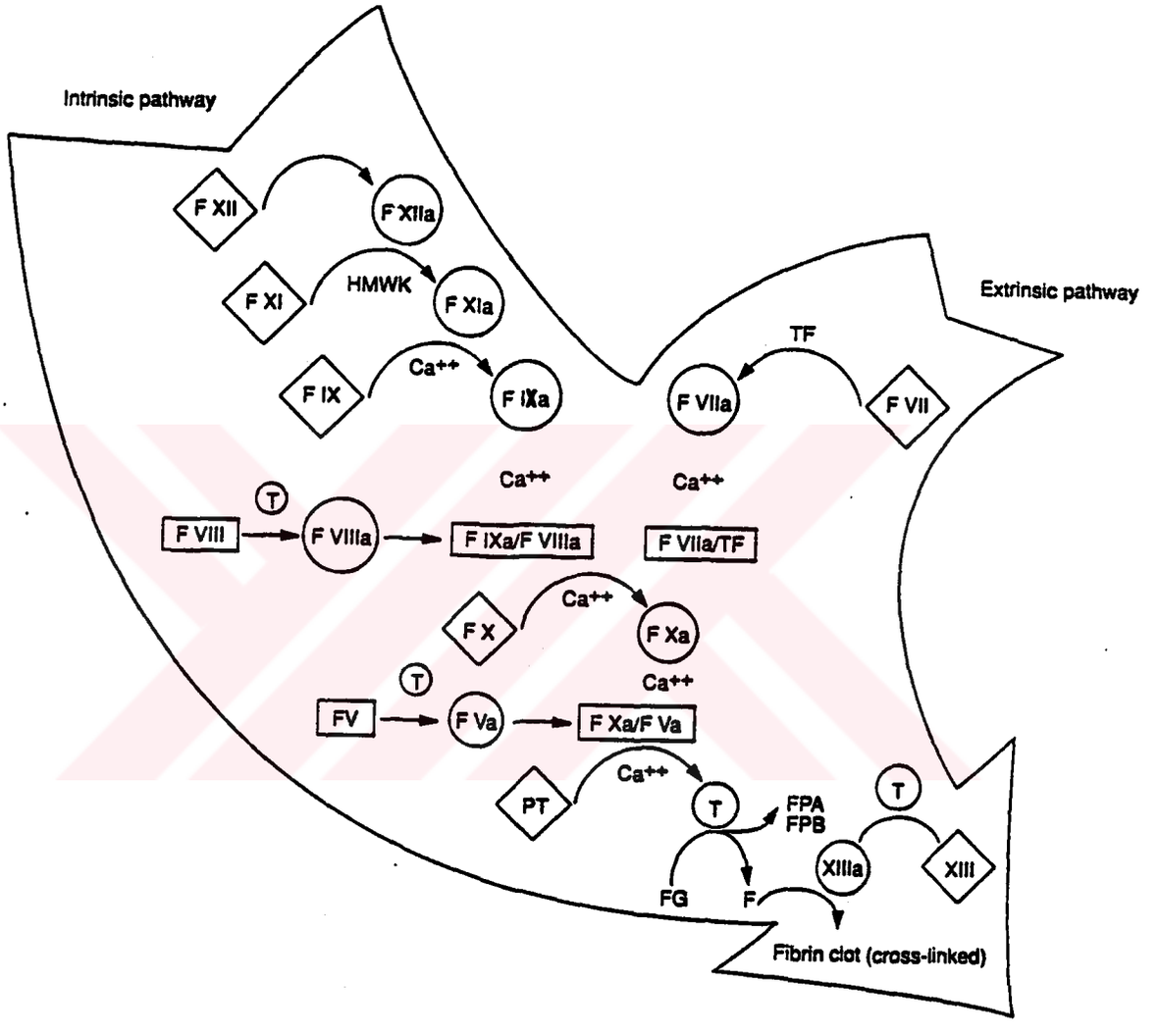

Figure I.1. The blood coagulation cascade. HMWK, high-molecular-weight-kininogen; TF, tissue factor; T, thrombin; FPA, fibrinopeptide A; FPB, fibrinopeptide B; FG, fibrinogen; F, fibrin clot (not cross-linked) (4).

B. Hemophilia A

The hereditary disorders of hemostasis consist of a number of different diseases. Hemophilia A (classic hemophilia, FVIII deficiency) and hemophilia B (Christmas disease, FIX deficiency) are relatively common inherited disorders of blood coagulation due to the deficiency or abnormality of FVIII and FIX, respectively. FVIII deficiency accounts for 85 per cent of cases while FIX deficiency explains 14 per cent and the remaining cases are the rare congenital clotting factor deficiencies such as deficiencies of FXI, FX, FVII, or FV (5).

Hemophilia A is an X-linked recessive disorder which means in practice that it is confined to males who inherit the abnormal bleeding tendency from their clinically normal mothers who are heterozygous carriers of the genetic defect. Daughters of carrier mothers have a 50 per cent risk of themselves being carriers; sons have a 50 per cent risk of being affected. Classic hemophilia afflicts one out of every 10,000 male births (6). Although affected female offspring of an affected father and carrier mother have been described, hemophilia A is rare in females. It may occur in females with X-chromosomal abnormalities such as Turner syndrome or other X-chromosomal defects (7, 8) and also due to Lyonization. There is no special ethnic group or geographic preference in this disorder.

The clinical diagnosis of a bleeding disorder depends on careful recording of family history, physical examination, and certain biochemical tests. Generally four screening tests identify the general locus of a hemostatic abnormality : prothrombin time (PT), partial thromboplastin time (PTT), thrombin time (TT), and fibrin stability test (9). They are available in most hospitals, inexpensive and simple to perform. These procedures reveal different patterns or syndromes. In the case of FVIII deficiency, PTT is abnormal, PT and other tests are normal. After the screening tests, specific factor assays (9) that are more expensive but highly accurate and available only in limited centers can be used to determine the deficient or abnormal coagulation protein.

Classic hemophilia is defined by excessive hemorrhage into various parts of the body. Hematomas and hemarthroses are highly characteristic of the disease (5, 6, 10). Hematomas are hemorrhages into subcutaneous connective tissues or into muscle groups, which is in the following order of frequency: calf, thigh, buttock, and forearm. They may lead to muscle contractures, nerve palsies and muscle atrophy. Hemarthroses are hemorrhages into joints that accounts for about 75 per cent of hemophilic bleeding (6). In order of decreasing frequency,

the joints most frequently involved include the knees, elbows, ankles, shoulders, wrists, and hips.

The severity of the clinical picture correlates with the extent of the clotting factor deficiency (11). FVIII can be expressed as per cent of normal clotting activity or in units per milliliter of plasma. By definition, one unit of FVIII per milliliter is 100 per cent (6). The disease is broadly classified as mild, moderate, and severe. Table I.2 shows a clinical classification based on both plasma FVIII concentration and the severity of the clinical manifestations.

The treatment of hemophilia A is achieved by replacement therapy which is intravenous infusion with FVIII. On the average each unit of FVIII infused per kilogram of body weight yields a two per cent rise in plasma FVIII level (5). The financial cost is in the range of $ 10,000 to $ 50,000 yearly per patient depending on body weight and severity. Since the half life of FVIII is eight to ten hours, twice daily infusions are needed to maintain a normal level at all times. In practice most patients are treated on demand and receive an infusion of FVIII at the earliest symptoms of bleeding (10).

At present, therapeutic FVIII is blood-derived (Table I.3) (10). Fresh-frozen plasma (FFP) and cryoprecipitate (crude FVIII concentrate) can be used (6, 10). Several commercial lyophilized FVIII concentrates, using cryoprecipitate of pooled normal human plasmas are available and do not have the disadvantages of plasma and cryoprecipitate. FVIII concentrates by monoclonal antibody techniques are highly purified and safer in terms of viral diseases (6). DDAVP (1-desamino-γ-D-arginine vasopresin), which is a synthetic analogue of vasopresin, was noted to cause a rise in FVIII and vWF levels without precursor effects (6, 10). Antifibrinolytic agents have also been used (6).

There are serious adverse side effects in the FVIII replacement therapy. Since FVIII concentrates are products made from plasma of between 2,000 to 30,000 donors, some complications occur including liver disease resulting from hepatitis B, and non-A, non-B hepatitis infections, and since 1978, transmission of the AIDS virus (HIV). But recently, virucidal treatment of concentrates has been developed (12, 13).

In addition to viral contamination, factor concentrate itself may cause development of antibodies (inhibitors) against FVIII in some hemophiliacs. The incidence of FVIII inhibitors is approximately five to ten per cent of all patients with hemophilia A and 10 to 15 per cent of those with severe hemophilia A (14).

The complications of FVIII replacement therapy have strengthened the demand for safer products. The cloning (15) and expression (16) of the FVIII gene is a promising step towards recombinant FVIII replacement therapy (17).

Table I.2. Clinical classification of hemophilia A.

Classification	FVIII:C level	Clinical features
Severe	< 1% of normal (< 0.01 U/ml)	1. Spontaneous hemorrhages from early infancy. 2. Frequent spontaneous hemarthroses and other hemorrhages. On average: 35 bleeds per annum.
Moderate	1-5% of normal (<0.01- 0.05 U/ml)	1. Hemorrhages secondary to trauma or surgery 2. Occasional spontaneous hemarthroses. Two or three bleeds per annum.
Mild	6-60% of normal (0.06-0.40 U/ml)	1. Hemorrhages secondary to trauma or surgery. 2. Rare spontaneous hemorrhage.

Table I.3. Therapeutic materials for treatment of hemophilia A (10).

Material	Factor VIII, units/ml	Advantages	Disadvantages
Fresh frozen plasma (FFP)	1	Low infection hazard	Storage at -20°C; high volume/low potency; allergic reactions
Cryoprecipitate	5-10	Low infection hazard (unless many units used)	Storage at -20°C; allergic reactions; potency not assayed
Heat-treated factor VIII concentrate	20-50	Assayed high potency, low or absent HIV and hepatitis B infectivity; storage at 4°C; few allergic reactions	Infective for non-A, non-B; high cost; heavy load of non-factor VIII proteins including iso anti-A, anti-B, β_2-microglobulin, fibrinogen, etc.
DDAVP	-	No infection risk; totally synthetic	Only effective in mild cases
Porcine* factor VIII (Hyate C)	20-50	No infection risk; high purity	Animal protein, allergic reactions; alloantibody to porcine factor VIII
Recombinant factor VIII	5000 units/mg	No infection risk; totally pure	Not yet available
Recombinant von Willebrand factor	0	N infectious risk; totally pure	Not yet available

*Generally reserved for treatment of inhibitor cases.

Gene replacement therapy (18, 19) is the ultimate cure of hemophilia A. Although considerable progress has been made towards developing gene therapy, no satisfactory experiments in animal models have been described yet (20).

In terms of surgery, replacement of knee, hip, and elbow joints is now possible. Normal livers have been transplanted successfully, however this procedure is still under development (6).

C. Factor VIII

Characterization of FVIII was hampered by its low concentration in plasma (0.1 μg/ml), its heterogeneity in size, and its sensitivity to degradation. Recent advances resulted from the use of immunoaffinity chromatography for successful purification of FVIII from porcine and human plasma, from the cloning of human FVIII gene and elucidation of the primary structure of FVIII (21).

The mRNA is synthesized in a variety of tissues including liver, spleen, lymph nodes, pancreas, kidney, muscle, placenta. Thus the sites of FVIII synthesis are diverse, although the hepatocyte is a major source of plasma FVIII (10).

1. Biochemistry of Factor VIII

FVIII is a large protein containing two functional entities. The major bulk of the protein which is precipitated by heterologous antisera is termed FVIII-related antigen (FVIIIR:Ag). FVIII:vWF is that part of FVIIIR:Ag which is associated with platelet-related activities. The smaller part of the FVIII complex is associated with the coagulant activity (FVIII:C) (11). It is linked to the FVIII:vWF molecule in a metal ion-dependent association (21). The antigenic determinance of FVIII:C are described as FVIII:CAg. It is the deficiency or abnormality of FVIII:C which is responsible for classical hemophilia (11). This antigen is measured as cross-reacting material (CRM) by a radioimmune assay

(22). Generally, the level of antigen is related with the level of FVIII activity in patients. For example, severely affected patients usually have no antigen (CRM^-). But in some severe hemophiliacs FVIII:CAg is present in large amounts, even though biological activity is not detectable (CRM^+), suggesting that the mutations have a very specific effect on function.

2. Structure of Factor VIII

Plasma FVIII circulates as a two chain, metal ion-stabilized complex which consists of a variable heavy chain of MW 90,000 to 210,000 and light chain of MW 80,000 (23). This complex is stabilized with a 50-fold excess of vWF (21).

The primary translation product is a single chain polypeptide of 2,351 amino acids. The 19 residues at the amino terminal comprise the signal sequence for FVIII (24). Therefore, the mature excreted polypeptide consists of 2,332 amino acids. The amino acid sequence reveales repeated blocks of amino acid homology between three types of structural domains that occur in the order A1:A2:B:A3:C1:C2 (Figure I.2) (21). A1:A2:B domains constitute the heavy chain and A3:C1:C2 domains form the light chain.

The three A domains are about 350 amino acids long, with 30 per cent homology to each other and to the A domains of ceruloplasmin (a copper-binding plasma protein), suggesting that the A domains may be involved in metal-ion-binding (Figure I.3) (21). The first repeat is separated from the second by 36 largely acidic amino acids. Following the second A domain repeat is a strech of 983 amino acids which is rich in potential asparagine-linked glycosylation sites. This region is the B domain and encoded by a single large exon (Exon 14). Another short segment of 41 predominantly acidic amino acids, which contains sequence required for binding of FVIII to vWF, is followed by the third A domain. Both copies of C domain are 150 amino acids long with 37 per cent homology between repeats and 20 per cent homology with discoidin I, a galactose-binding lectin, suggesting that the C domain may comprise the phospholipid-binding domain of FVIII (23). The amino acid sequences of FV and FVIII are 30 per cent homologous. This suggests that they have evolved from a primordial gene (21, 23).

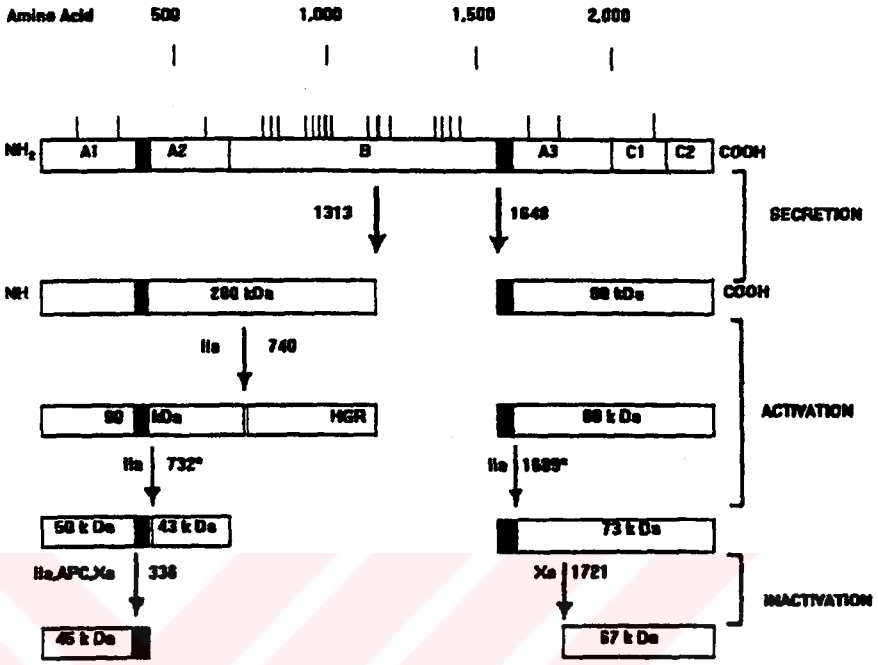

Figure I.2. Domain structure and processing of FVIII. The structural domains of FVIII include a triplicated A domain of 350 amino acids, a unique B domain of 983 amino acids, and a duplicated C domain of 150 amino acids. The black boxes represent regions with a high density of acidic amino acids. The vertical bars represent the position of potential asparagine-linked glycosylation sites. The thrombin (IIa), activated protein C (APC), and FXa cleavage sites are shown. FXa also cleaves at all the thrombin cleavage sites. The two cleavages required for thrombin activation are indicated by asterisks (21).

Figure I.2 shows the processing of FVIII. Intracellularly, FVIII is cleaved within the B domain to generate 200,000 dalton heavy chain and 80,000 dalton light chain. As a result of activation, the 200,000 dalton heavy chain is converted to 50,000 and 43,000 dalton polypeptides resulting in the complete loss of B domain and the 80,000 dalton light chain is converted to a 70,000 dalton polypeptide. Activation of FVIII also appears to involve the release of the acidic peptide of the light chain and separation of the first two A domain repeats, whereas inactivation occurs with the subsequent release of the second acidic peptide (21, 23, 25).

3. Structure of the Factor VIII Gene

Factor VIII gene is situated at the telomeric end of the long arm of the X chromosome at band Xq28. The entire gene comprises nearly 186 kb and constitutes about 0.1 per cent of the human X chromosome (26). It is divided into 26 exons ranging in size from 69 to 3,106 bp. The 25 introns range in size from 207 to 39,000 bp (23). FVIII mRNA is about 9,029 bp in length (26). Figure I.4 shows the exons coding for each protein domain in FVIII (25).

Intron sequences account for 95 per cent of the gene, and the largest of these, intron 22, is 39 kb in length. Preliminary analysis of this region revealed a high density of methylation-sensitive, GC-rich restriction enzyme sites suggesting that this intron might contain a CpG island, an unmethylated GC-rich sequence associated with some promoter regions (27). In fact, a gene inside intron 22 that is transcribed from the opposite DNA strand is found. The gene has a duplicate that lies outside the FVIII gene on the X chromosome. Because of its high level of transcription it may have a housekeeping role and may be a candidate for one of the many disease loci that have been mapped to Xq28 (28).

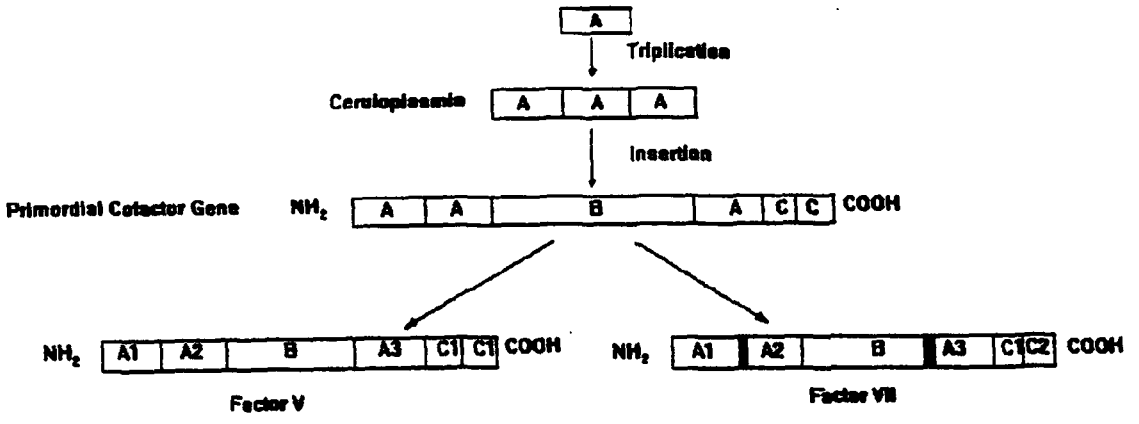

Figure I.3. Proposed evolution of coagulation factors V and VIII (21).

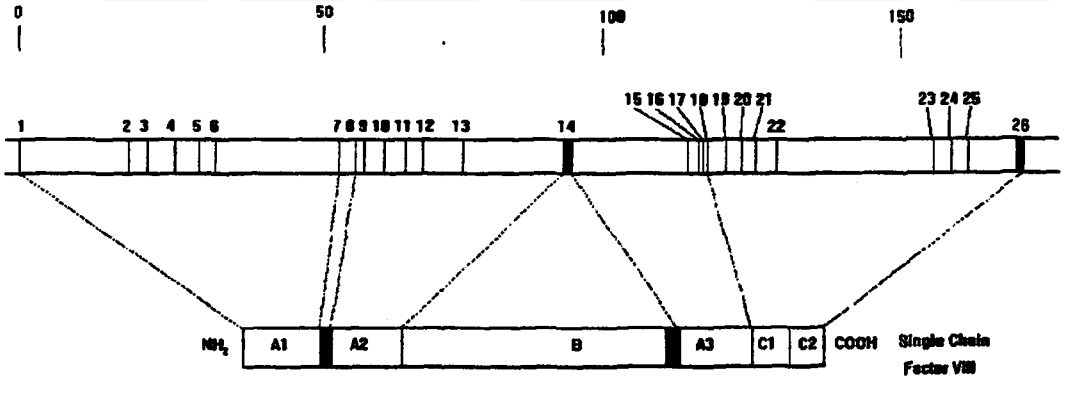

Figure I.4. Diagram of the FVIII gene (top bar) and FVIII protein (lower bar). Exons (1-26) are black boxes. Dotted lines indicate exon or group of exons coding for each protein domain (25).

D. Diagnosis of Hemophilia A

1. Biochemical Methods

Hemophilia A is assessed by phenotype on the basis of the following:

i) FVIII:C (Factor VIII coagulant activity): It is measured by one-stage clotting assays (29) and chromogenic substrate method (30).

ii) FVIII:Ag (Factor VIII antigen): It can be measured by various immunoradiometric (IRMA) or enzyme-linked immunosorbent (ELISA) assays (31). The measurement of FVIII:Ag is diagnostic only in CRM^- families and can not be used in CRM^+ families (23).

iii) vWF:Ag (von Willebrand factor antigen): It can be measured quantitatively with two different methods: electroimmunoassay (EIA) and IRMA or ELISA (32).

Carriers of hemophilia A have about 50 per cent of the normal level of the FVIII. However, owing to the wide range of normal values (50-150 per cent), a carrier will often be within the normal range. By using the ratio of FVIII:C to vWF:Ag, it is possible to improve discrimination, since carriers generally have an excess of vWF:Ag over their FVIII:C when compared to normal subjects (10).

Biochemical methods used in carrier detection is also subject to the level of Lyonization, age and blood type, severity of hemophilia, pregnancy, use of oral contraceptives and probably other factors that influence the accuracy of the diagnosis (33).

Prenatal diagnosis by biochemical methods is not feasible since FVIII levels in the fetal plasma can only be determined on samples obtained in mid-gestation by ultrasound guided, transabdominal umbilical vein puncture (cordocentesis) (34). At this stage, fetal plasmas average about half the normal adult levels of FVIII:C. The risk of fetal death is one to two per cent (20).

2. DNA-based Methods

DNA based methods allows for the identification of carriers and prenatal diagnosis with greater certainty than biochemical methods.

The two major molecular techniques based on DNA analysis are those of Southern blotting (35) and the polymerase chain reaction (PCR) (36, 37). The former method has been in use for more than a decade and involves a relatively labour-intensive schedule comprising the capillary transfer of restriction endonuclease-digested DNA fragments from an agarose gel to a membrane support and the subsequent probing of the membrane with a radiolabelled DNA fragment representing the sequence of interest (38). It requires a minimum of five μg of DNA for testing and takes at least five to seven days to obtain the results. Small deletions, insertions, and point mutations are only detectable by Southern blotting if the mutation alters the site of a restriction enzyme. This approach does not detect the majority of mutations (25). It has been applied to hemophilia A diagnosis by using cDNA probes that detected various large deletions (38).

PCR has now replaced Southern blotting in many instances. This technique utilizes synthetic oligonucleotide primers that recognize flanking regions of the sequences of interest which are then amplified in vitro a millionfold of their original concentration. The power of this method includes the ability to detect all types of mutations, work with very small starting quantities of DNA (less than one μg), simplicity, pure and high yield for all further methods of analysis such as restriction enzyme digestion and sequencing. The only drawback of PCR is the requirement of sequence information, at least partially, of the gene of interest.

a. Diagnosis by DNA Linkage Analysis

Two approaches have been used in molecular diagnosis of hemophilia A: DNA linkage analysis and direct detection of mutations.

Hemophilia A mutations are heterogenous and in most instances involve changes of single nucleotides. Moreover, the size and the complexity of the FVIII gene possess a challenge to the direct detection of mutations. As a result, the majority of diagnostic studies rely upon indirect gene linkage testing.

Indirect diagnosis relies on the identification of variations in the DNA sequences (polymorphisms) within or in the vicinity of the gene of interest and which are subsequently inherited in a Mendelian manner with the mutant gene. Therefore they may be used to track the mutant gene by means of linkage analysis.

Restriction fragment length polymorphisms (RFLPs) represent nucleotide sequence variations that occur throughout the genome in every individual. Their overall frequency is approximately one nucleotide change in 300, although there is significant regional variability in their occurrence in the genome (38). The most frequent and simple examples are the bi-allelic polymorphisms resulting from single nucleotide substitutions which either create or abolish restriction endonuclease sites. Their maximum level of heterozygosity is 50 per cent (20). In addition, there are other polymorphic loci in the genome that can be shown to be multi-allelic in nature and that represent the presence of variable numbers of a repeated nucleotide sequence. These so-called variable number of tandem repeat (VNTR) loci are especially useful in linkage studies because of higher heterozygosity than with bi-allelic polymorphisms.

Although DNA-based diagnosis by RFLPs is relatively accurate, allows first trimester testing and is not affected by Lyonization, there are several problems and limitations:

1) Sometimes the affected person or some crucial family member is not available to donate DNA.

2) Non-paternity or other inaccuracies in the pedigree can lead to errors.

3) Sporadic cases of hemophilia comprise 30-50 per cent of the total hemophilic population. In these cases, polymorphism analysis can only be used to exclude carrier status (39).

4) Another genetic locus may account for the clinical picture (disease heterogeneity).

5) The presence of heterozygosity or informativeness in females for a polymorphism must be satisfied to track individual copies of the FVIII gene.

6) The polymorphic site should either be within the gene (intragenic) which disease is segregating or close enough (extragenic) to ensure that the possibility for genetic recombination between the polymorphism and mutation is minimal. For an intragenic RFLP, the probability of such a recombination (40,41) is usually less than 0.1 per cent per meiosis. The uncertainty of diagnosis is estimated to be two per cent (40). When an extragenic marker is used its usefullness will depend on the tightness of the linkage (41) and the answers will be probabilistic with a four to ten per cent uncertainty of diagnosis (40).

7) The closely linked polymorphisms can be in linkage disequilibrium (41).

Figure I.5 shows the sites of common polymorphic sequences in the FVIII gene (20) and tables I.4 and I.5 list some FVIII intragenic polymorphisms, their heterozygote and allele frequencies in Caucasian (42-50) and Turkish population (51), respectively. There is a strong linkage disequilibrium between the *Bcl* I, *Hind* III and *Bgl* I sites. In contrast, the *Xba* I is often informative in females who are homozygous for the *Bcl* I site. Intron 7 is useful in some cases where the *Bcl* I and *Xba* I polymorphisms are not informative (43). Two multi-allelic intragenic polymorphisms are CA dinucleotide tandem repeats that occur within introns 13 and 22. The Intron 13 repeat with eight alleles has a heterozygosity rating of approximately 80 per cent (20).

In some cases, no informative intragenic polymorphisms can be identified and extragenic sites must be used (52, 53). Table I.6 lists FVIII extragenic polymorphisims (54-58). The two extragenic RFLPs, *Taq* I/St 14 and *Bgl* II/DX 13 are both closely linked to the FVIII gene. The St 14 probe detects several polymorphisms at the DXS52 loci, including a VNTR with at least 12 alleles revealed by *Taq* I. It specifically maps to Xq28 and is about two cM from the FVIII gene (57, 58). The alleles are duplications of 60 bp repeat with a minimum size of approximately 600 bp. PCR primers are available that amplify the 14 alleles of the VNTR region at DXS52 (58). Twelve alleles in Turkish population have been detected by PCR (Table I.5) (51).

b. Direct Diagnosis

The heterogeneity of the hemophilia A mutations requires the detailed analysis of the essential regions of the gene in every unrelated case in order to detect the causative mutation. This comprises all exons; intron-exon boundaries including sequences involved in branchpoints, promoter and 5' flanking sequences which may be involved in control of transcription; and AATAAA and other 3' untranslated sequences which are involved in termination and maturation of mRNA.

The use of direct sequencing is effective as sequence data give complete information about the mutation. The ability to detect both alleles in a heterozygote by direct sequencing (59) makes this approach applicable to carrier detection and prenatal diagnosis. However, due to the large size of the FVIII gene, direct sequencing is not feasible. Instead screening methods are

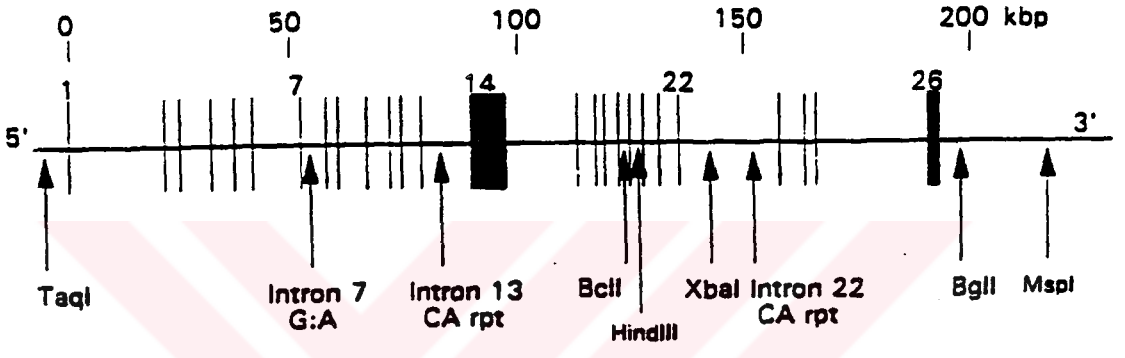

Figure. I.5. Line diagram of the factor VIII gene showing the sites of common polymorphic sequences identified (20).

Table I.4. Factor VIII intragenic DNA polymorphisms.

Locus	Restriction enzyme	Testing method (PCR or southern blot)	Heterozygosity (%)	Southern blot alleles (kb)	PCR alleles (bp)	Allelic frequencies (%) (in Caucasian)	References
5' flanking region	*Taq* I	Blot	40	9.5	-	72	42
		Probe: P 701.1		4.0	-	28	
Intron 7	-	PCR and ASO probes	32	-	Guanine	79	43
					Adenine	21	
Intron 7	*AlwN* I	PCR	33	-	260	79	43
				-	230	21	
Intron 13	-	PCR (CA repeat)	91	-	CA repeat 8 alleles	1 to 45	44
Intron 18	*Dcl* I	PCR and blot	42	1.1	142	29	45
		Probe: P 114.12		0.88	99+43	71	
Intron 19	*Hind* III	PCR and blot	42	2.7	504+234	70	46,47
		Probe: p 114.12		2.6	504+157+77	30	
Intron 22	*Xba* I	PCR and blot	48	6.2	96	41	48
		probe: p 482.6		4.8+1.4	68+28	59	
Intron 22	-	PCR (CA repeat)	44	25 repeats	CA repeat	33	45,48
				26 repeats		67	
3' of exon 26	*Bgl* I	Blot	18	20	-	10	49
		probe: FVIII cDNA-C		5	-	90	
3' of exon 26	*Msp* I	Blot	44	7.5	-	68	50
		probe: p 625.3		7.3+3.2	-	32	

Table I.5. Allelic and heterozygote frequencies of FVIII polymorphisms in individuals of Turkish origin. The first column of allele frequency shows the number of X chromosomes analyzed for each RFLP. EHF, OHF and CHF denote the expected, observed and cumulative heterozygote frequencies, respectively (51).

		Allele Frequency		Heterozygote frequency (%)		
Marker	**Alleles**	**#**	**%**	**EHF**	**OHF**	**CHF**
Hind III	+	105/235	45	49.5	41	41
	-	130/235	55			
*AlwN*I	+	51/56	91	16.4	11	45
	-	5/56	9			
St14	2700	1/74	1.4			
	2400	1/74	1.4			
	2100	2/74	2.7			
	1810	9/74	12			
	1750	9/74	12			
	1690	5/74	6.8			
	1630	2/74	2.7			
	1570	7/74	9.4			
	1390	9/74	12			
	1300	14/74	19			
	1220	2/74	2.7			
	700	13/74	18			

Table I.6. Factor VIII extragenic DNA polymorphisms.

Locus	Restriction enzyme	Testing method (PCR or Southern blot)	Heterozygosity (%)	Southern blot alleles (kb)	PCR alleles (bp)	Allelic frequencies (%) (in Caucasian)	Recombination (%)	References
DXS15	*Bgl* II	Blot probe: DX13	50	5.8 2.8	- -	50 50	5	54
DXS115	*Msp* I	Blot probe: 767	24 24	11.8 6.0+5.8	- -	86 14	?	55
DXS115	*Ace* I	Blot probe: 767	18	9.0 4.0	- -	10 96	?	55
DXS115	*Pst* I	Blot probe: 767	35	1.8 1.75	- -	77 23	?	56
DXS115	*Bst* XI	Blot probe: 767	24	6.4 4.25	- -	84 14	?	57
DXS52	*Taq* I/*Msp* I	PCR and blot probe: st14	>70	multiallelic	60 bp repeat; 14 alleles	-	5	58

used to locate the possible change in the base composition. Four screening methods have been used for Hemophilia A in search for the causitive mutation prior to sequencing.

i) RNase A cleavage: This method (60) involves reassociating RNA that is complementary to the normal gene to DNA from the patient. A sequence difference results in formation of a mismatch in the RNA-DNA duplex at which is susceptible to RNase A cleavage. The length of the fragments that are generated by this treatment indicates the sites of mutation.

ii) Chemical cleavage mismatch detection (CCD):This method (61) results in partial cleavage of the DNA fragment at the site of the mutation, so giving both positional information and allowing the simultaneous definition of multiple sequence alterations in a single fragment.

iii) Single-strand conformational polymorphism (SSCP): This method (62) is based on the principle that under non-denaturing conditions, single-stranded DNA takes up a conformation which is stabilized by intra-strand interactions. Sequence differences between two samples are reflected by a difference in conformation and hence mobility of the single-stranded DNA in non-denaturing gels.

iv) Denaturing gradient gel electrophoresis (DGGE): Higuchi and colleagues (63, 64) have applied this method. Electrophoresis of DNA duplexes in a gradient of increasing denaturants results in denaturation of the duplex and marked reduction of mobility of the strands at a characteristic position in the gel which is specific to each fragment. A single base change in the fragment is sufficient to alter the melting characteristics of the duplex in a proportion of cases such that a band shift is observed on the denaturing gradient gel. Each fragment is considered to have domains of different melting properties (65, 66, 67). By adding an artificially GC-rich region to one of the primers used in the PCR, the sequence variations in the highest temperature melting domains can be detected (68).

These techniques use PCR-amplified DNA as their test material. A recent study indicates that ectopically transcribed FVIII mRNA from blood leukocytes can be used as the template for PCR of FVIII sequences (69).

E. Mutations Causing Hemophilia A

A compilation of mutations in the FVIII gene updated to August 1991 has been published in Nucleic Acids Research (70); over 80 different point mutations, six insertions, seven small deletions, and over 60 large deletions are catalogued.

i) Nucleotide substitutions: All nonsense mutations result in severe hemophilia. All examples of point mutations causing moderate and mild hemophilia are of the missense type. The clinical phenotype with the same point mutation is not always the same. This may be due to the epistatic effects of other loci on the expression of FVIII. Ten out of twelve inhibitor patients possess a nonsense mutation and exhibit a severe phenotype. There may be some association between specific mutations and the presence of inhibitors. CRM (71) status is often selected for study in the hope of gaining information on the functionally critical regions of the FVIII protein; so that one can distinguish mutations affecting protein stability from those with a critical effect on protein function (70). Thirty eight percent of point mutations are C->T or G->A transitions in a CpG dinucleotide (72). This doublet is already known to be hypermutable as a result of methylation-mediated deamination of 5-methyl cytosine.

ii) Deletions: Over 60 different total or partial deletions of the FVIII gene have been reported. Partial deletions vary in size from one bp to over 200 kb. All but two are associated with severe phenotypes. Although there is no evidence for deletion hotspots, deletion of the FVIII gene seems to have a five-fold higher risk of developing inhibitors compared with other severe hemophiliacs without gene deletions (70).

iii) Insertions: Two unrelated patients with severe hemophilia have insertions of L-1 repetitive elements into their Exon 14 (73). Four examples of the insertion of a single base (three of them A residues) have also been noted. The introduction of an A into an existing string of A residues is consistent with slipped mispairing at the replication fork (70).

iv) Duplications: Duplications are highly unusual. A 23 kb duplication of Intron 22 and an in-frame duplication of Exon 13 (74) have been described.

v) Mutations affecting mRNA splicing: The putative splicing defects reported to date may be divided into three categories: mutation of the invariant GT and AG dinucleotides at the donor and acceptor splice junctions respectively, mutation within the extended consensus sequences of the donor and acceptor splice junctions, and mutations which create a novel donor or acceptor splice

site (70).

There are no examples of mutations in the FVIII promoter region reported to date.(70).

II. PURPOSE

Hemophilia A is the most common bleeding disorder caused by the deficiency or lack of the coagulation protein factor VIII. The FVIII gene is located on the X-chromosome and known to be one of the most complex and large genes sequenced to date.

Molecular basis of hemophilia A is extremely heterogenous and point mutations account for 95 per cent of the molecular defects. Treatment of hemophilia A is costly and there is no absolute cure yet. Therefore, prevention of the disease is very important. Prevention of X-linked disorders is possible if phenotypically normal carriers can be identified accurately and diagnosis of affected fetuses can be carried out in early fetal development. Biochemical methods are not sufficiently sensitive for accurate diagnosis of hemophilia A. However, the introduction of DNA-based methods has radically improved its accurate diagnosis either by linkage analysis or direct detection of mutations.

Direct detection of mutations as well as providing accurate carrier determination and prenatal diagnosis also reveals the functionally important regions of the protein product, new mechanisms of mutations, rearrangements within genes and their detailed structure. However, the size and complexity of the FVIII gene hampers the direct identification of the point mutations. A number of rapid screening methods have been used to overcome these factors and make this approach feasible for such large genes. Consequently, we have chosen Denaturing Gradient Gel Electrophoresis (DGGE) to screen point mutations in three exons of the FVIII gene of 78 hemophilia A patients to initiate the identification of causative mutations in the Turkish population.

The second aim of this thesis was to carry out linkage analysis studies in families afflicted with hemophlia A since it is still the widely used DNA-based method for prenatal diagnosis and identification of carriers. In this context, we aimed at establishing the use of a multi-allelic intragenic site in molecular diagnosis of hemophilia A in Turkey.

III. MATERIALS

A. Blood and Fetal Samples

Blood, amniotic fluid or chorionic villi samples from hemophilia A patients and their family members were obtained from the Department of Hematology, Cerrahpaşa Medical School, Istanbul University, Istanbul; Department of Pediatric Hematology, Medical School, Hacettepe University, Ankara; Department of Pediatric Hematology, Medical School, Gazi University, Ankara; Pretam, Istanbul Medical School, Istanbul University, Istanbul; Department of Pediatrics, Medical School, Ege University, Izmir, and Gülhane Military School of Medicine, Ankara.

B. Chemicals

All chemicals and solutions used in this study were purchased from MERCK (Germany) or SIGMA (USA) unless stated otherwise in the text. Absolute alchohol was from TEKEL (Turkey).

C. Oligonucleotide Primers:

All FVIII specific primers used in DNA linkage analysis and DGGE except the following two were purchased from MAM-TÜBİTAK (TURKEY).

St14 and Int13/$(CA)_n$ repeat specific primers were purchased from BIOMETRA (GERMANY).

The sequences for all the oligonucleotide primers used in this study are given in Appendix A.

D. Enzymes

The enzyme *Taq* DNA polymerase was from PROMEGA (USA), Cetus Corporation (USA), and BOEHRINGER MANNHEIM (Germany).

The restriction enzymes with their appropriate reaction buffers were from PROMEGA, BOEHRINGER MANNHEIM, and NEW ENGLAND BIOLABS (USA).

The enzyme T4 polynucleotide kinase was from PROMEGA.

E. Buffers and Solutions

1. DNA Isolation Buffers

a. For DNA Extraction from Whole Blood

Lysis Buffer : 155 mM NH_4Cl,
10 mM $KHCO_3$,
0.1 mM EDTA

Nuclei Lysis Buffer : 10 mM Tris-HCl (pH 8.0),
400 mM NaCl,
2 mM Na_2 EDTA (pH 8.2)

SE Buffer	: 75 mM NaCl, 25 mM Na_2 EDTA, 30 mM Tris-HCl (pH 8.0)
Sodium dodecyl sulfate (SDS)	: 10% stock solution
Proteinase K	: 20 mg/ml in H_2O
Saturated NaCl	: 5 M NaCl
Phenol/chloroform	: 50mM TE saturated phenol and chloroform (1:1)
Sodium acetate	: 3 M stock solution
TE Buffer	: 20 mM Tris-HCl (pH 8.0), 0.1 mM Na_2EDTA (pH 8.0)

b. For DNA Extraction from Chorionic Villi or Amniotic Fluid

NaCl/EDTA Lysis Buffer	: 100mM NaCl, 125 mM Na_2EDTA
SDS	: 10% stock solution
Proteinase K	: 20 mg/ml in H_2O
Chloroform/isoamylalcohol	: 24:1 chloroform/isoamylacohol
Ammonium acetate	: 7.5 M stock solution

2. Polymerase Chain Reaction Buffers

Guy's Buffer (1X)	: 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 0.17 mg/ml BSA, 10 mM β-Mercaptoethanol, made up to a final volume with T0.1E (20 mM Tris, 0.1 mM Na_2EDTA)
Boehringer Mannheim Buffer (1X)	: 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl
Promega Mg^{2+} free Buffer (1X)	: 100 mM Tris-HCl (pH 9), 500 mM KCl, 1.0 % Triton X-100

3. Restriction Enzyme Digestion Buffer

*Hind*III Enzyme Buffer	: 500 mM NaCl, 100 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM DDT (pH 7.9)
*Alw*NI Enzyme Buffer	: 50 mM K-acetate, 20 mM Tris-acetate, 10 mM Mg-acetate, 1 mM DDT (pH 7.9)

4. Electrophoresis Buffers and Gel Systems

a. Electrophoresis Buffers

5X TBE (Tris-Borate) Buffer	: 445 mM Tris-base, 445 mM Boric acid, 10 mM Na_2EDTA
20X TAE (Tris-Acetate) Buffer	: 0.8 M Tris-base, 0.4 M Sodium acetate, 0.02 M Na_2EDTA (pH 7.4)
10X Loading Buffer A	: 2.5 mg/ml BPB, 1 % SDS in glycerol
10X Loading Buffer B	: 80 % Formamide, 0.05 % BPB, 0.05 % XC, 1 mM Na_2EDTA, 10 mM NaOH
10X Loading Buffer C	: 20% Sucrose, 10 mM Tris (pH 7.8), 1 mM Na_2EDTA, 0.25% BPB, 0.25% XC
Stop/Loading Buffer	: 0.3% XC,0.3% BPB,0.37% Na_2EDTA (pH 7.0) in deionized Formamide
Ethidium Bromide	: 10 mg/ml

b. Gel Systems

Agarose gel	: Agarose in 0.5X TBE or in 1X TBE
NuSieve	: 2% NuSieve, 1% Agarose in 0.5X TBE
30% Acrylamide (19:1)	: 29% Acrylamide, 1% Bisacrylamide
40% Acrylamide (19:1)	: 38% Acrylamide, 2 % Bisacrylamide
Instagel Solution I	: 6% Acrylamide/Bisacrylamide (19:1), 6.3 M Urea, 1X TBE Buffer (pH 8.3)
Instagel Solution II	: 8% Acrylamide/Bisacrylamide (19:1), 6.3 M Urea, 1X TBE Buffer (pH 8.3)
Instagel Solution III	: 8% Acrylamide/Bisacrylamide (19:1), 7 M Urea, 30-40% Deionized Formamide, 1X TBE Buffer (pH 8.3)
40% Acrylamide (37.5:1)	: 37.5% Acrylamide, 1% Bisacrylamide
0% Stock Denaturing Gel	: 8-12% Acrylamide/Bisacrylamide (37.5:1), 1X TAE Buffer (pH 7.4)
80% Stock Denaturing Gel	: 8-12% Acrylamide/Bisacrylamide (37.5:1) 5.6 M Urea, 32% Deionized Formamide, 1X TAE Buffer (pH 7.4)

10% Ammonium peroxidisulphate (APS)	: 1 g APS in 9 ml H_2O

5. End-Labeling Buffer

10X Polynucleotide kinase Buffer	: 500 mM Tris (pH 7.4), 100 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine
Spermidine	: 40 mM spermidine stock

F. Equipment

Autoclave	: Medexport, USSR
Balances	: Precision Balance H72, METTLER, Germany Electronic Balance Type 1574, SARTORIUS, Germany Electronic Balance Libror EB-3200H, SHIMADZU, Japan
Camera	: RB67, MAMIYA, Japan
Centrifuges	: SORVALL RC-5B Refrigerated Superspeed Centrifuge, DUPONT, USA Biofuge A, HERAUS CHRIST, Germany Hettich EBA 3S, Germany
Deepfreezes	: -70, GFL, Germany -20, BOSCH, Germany

Electrophoresis	:	DGGE Apparatus, PS500 XT, HOEFFER SCIENTIFIC INSTRUMENTS, USA Horizon 58, Model 200, Horizontal Gel Electrophoresis Apparatus, Model H1 and H5, Sequencing Apparatus, Model S2, BRL, USA Miniprotean II, BIORAD, England
Freeze-Drier	:	ChemLab Instruments Ltd, Model SB6, England
Heat-block	:	Multi-block LAB-LINE, USA
Incubators	:	Incubator, Plus Series, GALLENKAMP, Germany Oven 300, Plus Series, GALLENKAMP, Germany
Power Supplies	:	ECPS 3000/150 Constant Power Supply, PHARMACIA, Sweden Model 100, BRL, USA
Spectrophotometer	:	Lambda 3 UV/VIS, PERKIN-ELMER, CETUS, USA
Thermo-cycler	:	Model 480, PERKIN-ELMER, CETUS, USA
Transilluminator	:	Reprostar II, CAMAG, Switzerland
Water baths	:	Thermomix, BU, BRAUN, Germany Thermomix 1441, BRAUN, Germany

G. Others

Circumvent™ Thermal Cycle Dideoxy DNA Sequencing Kit was from NEW ENGLAND BIOLABS.

*Hind*III digested lambda phage DNA, *Hae*III digested ϕ–X174, deoxyribonucleotides (dNTPs), nuclease-free water were from PROMEGA.

The radio nucleotides α-[^{35}S]-dATP (1000 Ci/mmol) and γ-[^{32}P]-dATP (6000 Ci/mmol) were from NEN-DUPONT (USA).

Centricon spin dialysis membranes were from AMICON (USA).

Instant films (Polaroid 667) were from POLAROID (USA).

X-ray films (XAR-5) and the developing solutions were from KODAK (USA).

IV. METHODS

A. DNA Extraction

1. DNA Extraction from Whole Blood

10 ml of peripheral blood samples collected into vacutainer tubes containing K_2EDTA to prevent coagulation were either kept at 4°C for a few days or at -20°C or -70°C until DNA isolation.

The blood samples were allowed to thaw and transferred to 50 ml Sorvall polypropylene centrifuge tubes. 30 ml ice-cold lysis buffer were added to the samples and mixed well. The samples were left on ice or kept at 4°C for 15 min to allow lysis of erythrocyte membranes. The lysis was completed when sample became transparent. The lysed mix was spun at 5,000 rpm and 4°C for 10 min to collect leukocyte nuclei. The pellet was washed in 10 ml lysis buffer at 5,000 rpm for 10 min (The nuclei can be stored at -70°C until DNA extraction). The pellet was treated according to the salt extraction or the phenol/chloroform extraction method.

a. Salting Out (NaCl) Extraction Method

The nuclear pellet was resuspended in 3 ml nuclei lysis buffer to break the nuclear envelope and vortexed until the clumps completely disappeared. Proteinase K (150 μg/ml) and SDS (0.14 per cent) were added, and the lysate was gently mixed by rolling the tube several times. The mixture was incubated at 56°C for 3 h or at 37°C for overnight. After the incubation, 5 ml of water and 5 ml of saturated NaCl were added. The tube was shaken vigorously to precipitate proteins. The sample was then centrifuged at 10,000 rpm at room temperature for 20 min. The supernatant containing the DNA was precipitated with 2 volumes of absolute ethanol.

b. Phenol/Chloroform Extraction Method

The nulear pellet was resuspended in 4.5 ml of SE buffer by vortexing until all the clumps were broken. Proteinase K (100 μg/ml) and SDS (1%) were added and the lysate was gently mixed by rolling the tube. The mixture was incubated at 56°C for 3 h or at 37°C for o/n. In this step, the nuclear proteins were degraded and prevented from carrying the DNA into the phenol phase in the next step. 5 ml of SE buffer and 10 ml of phenol/chloroform (1:1) were added to the lysate to remove the proteins and the suspension was shaken vigorously. After centrifugation at 5,000 rpm at rt. for 5 min, the upper aqueous phase containing the DNA was re-extracted with phenol/chloroform. DNA was precipitated by the addition of 1/3 volume of 3 M ammonium acetate (pH 5.5) and 1 volume of isopropanol.

At the end of each method the DNA was fished out, dried and kept at rt. to dissolve completely in a suitable amount of TE buffer.

All centrigations were carried out in Sorvall RC-5B refrigerated high speed centrifuge using an SS34 rotor, and all solutions and glassware were sterelized before use.

2. DNA Extraction from Chorionic Villi or Amniotic Fluid

The sample containing either chorionic villi or amniotic fluid was centrifuged to collect the cells and the supernatant was decanted. Then 0.5 ml NaCl/EDTA lysis buffer, 0.1% SDS and proteinase K (50 μg/ml) were added and mixed well to lyse the cells. The suspension was incubated at 56°C for 3 h or at 37°C for o/n. After incubation the nuclei were treated with 1 volume of chloroform/isoamylalcohol (24:1) and 1 volume of phenol two times to remove the protein debris. In order to increase the purity of the DNA, chloroform/isoamylalcohol (24:1) extraction was performed twice. DNA was precipitated by adding 3.75 M ammonium acetate and 2 volumes of ethanol. The DNA was dissolved in 10 to 100 μl of sterile distilled water depending on the amount of DNA.

3. Quantitative and Qualitative Analysis of the Extracted DNA

There are two methods in the quantitative and qualitative analysis of the extracted DNA: spectrophotometric method and minigel method.

a. Spectrophotometric Method

The optical density of the dissolved DNA was read at 260 nm and its concentration was calculated by means of the formula

$$50\ \mu g/ml \times OD_{260} \times \text{dilution factor} = \text{concentration in } \mu g/ml$$

since 50 μg of dsDNA has an absorbance of 1.0 at 260 nm (OD_{260}).

The purity of the DNA was estimated by taking the ratio of the optical densities at 260 nm to 280 nm (OD_{260}/OD_{280}). A pure sample is indicated by a value of 1.8.

b. Minigel Method

The minigel was prepared by using 0.8 per cent agarose (w/v) in 0.5X TBE buffer containing ethidium bromide (0.5 μg/ml) to visualize DNA under the UV light. One to two μl from each DNA sample were mixed with 1X loading buffer A in a total volume of 8-10 μl and loaded into the slots of the gel. The gel was electrophoresed at 100-150 V in 0.5X TBE buffer. The amount of DNA was estimated by comparing its intensity with known amounts of DNA (75).

B. Restriction Fragment Length Polymorphism (RFLP) Analysis

*Hind*III (Intron 19), *Alw*NI (Intron 7), and St14 (DXS52) polymorphisms were used for the linkage analysis.

1. Polymerase Chain Reaction (PCR)

50 μl reaction volumes contained 1X Guy's buffer, 1 mM of each of the four dNTPs, 0.2 μM of each primer, 0.5-1.0 μg of genomic DNA and 2.5 units of *Taq* polymerase. Samples were layered with 2 drops of mineral oil to prevent evaporation.

The following program was used to amplify (36, 37) *Hind*III and *Alw*NI RFLP sites. Two step cycles consisted of a 1 min incubation at 92°C and a 4 min incubation at 60°C for annealing of the primer to target sequences and for synthesis followed a 7 min incubation at 92°C for initial denaturation of the target DNA. 30 cycles were followed by a final incubation of 7 min at 60°C for completing the extension of some unfinished chains.

For St14 locus each PCR cycle consisted of 2 min at 94°C, 2 min at 60°C and 9 min at 72°C which was repeated 30 times. The final incubation was 30 min at 72°C.

The sets of primers used for the analysis of the RFLPs are summarized in Appendix A.1.

To check whether sufficient amplification has occured, 5 μl aliquots of *Hind*III PCR products were mixed with 1 μl 10X loading buffer A and electrophoresed on 1.2 per cent agarose gels. 5 μl aliquots of *Alw*NI PCR products were electrophoresed on either 1.8 per cent agarose gels or a 6-12 per cent polyacrylamide gel which was stained by EtBr after electrophoresis.

25-30 μl of St14 PCR products were analyzed directly on 1 per cent agarose gels in 1X TBE buffer. λ/*Hind*III and ϕ-X174/*Hae*III were used as standard DNA size markers (see Appendix B).

2. Restriction Enzyme Analysis

A 10-15 μl aliquot of the amplified DNA was digested with 15-20 units of the appropriate restriction enzyme and its 1X reaction buffer in a 20 μl reaction volume. The mixture was incubated at the *Hind*III and *AlwN*I specific temperature, which is 37°C for 3 h. There was no RE analysis for St14 locus since the VNTR alleles were directly examined on gels.

10 μl digested product of the *Hind*III site was electrophoresed on 1.8 per cent agarose gel and 10-15 μl of the *AlwN*I digested product was separated on 12 per cent polyacrylamide gel.

In all electrophoresis, the concentration of the buffer in the gel and electrophoresis tank was the same. The electrophoresis was carried out at 100-150 V in all cases.

C. Analysis of the Hypervariable Dinucleotide Repeat in Intron 13 of the FVIII Gene

1. Polymerase Chain Reaction (PCR)

The forward PCR primer Int-13A (see Appendix A.1) was end-labeled with γ-[^{32}P]-dATP. Thirteen μl total volume contained 1X end-labeling buffer, 0.1 μg of oligonucleotide, 7.5-10 units of polynuceotide kinase and 100 μCi γ-[^{32}P]-dATP (6,000 Ci/mmol). The mixture was incubated for 30 min at 37°C and then for 5 min at 60°C. Seven μl of water was added for a final concentration of 5 ng of oligonucleotide per μl.

The hypervariable loci at Intron 13 involving a strech of $(CA)_n$ repeats was amplified in a total volume of 12.5 μl reaction volumes that contained 1X Boehringer Mannheim PCR reaction buffer, 0.1 mM of each of the four dNTPs, 75 ng of each primer, 5 ng of end-labeled primer, 0.5 unit of Taq polymerase, and 0.5-1.0 μg of genomic DNA. Samples were layered with one drop of mineral oil and were subjected to the following PCR conditions.

The initial denaturation step was at 94°C for 5 min. Each cycle consisted of a 35 sec incubation at 94°C, a 1 min incubation at 52°C and a 1 min incubation at 72°C. Thirty five cycles were followed by a final incubation for 5 min at 72°C.

After PCR, The samples were lyophilized and dissolved in 5-10 μl of loading solution B (44).

2. Electrophoresis and Autoradiography

The PCR products were subjected to electrophoresis on 0.4 mm thick sequencing gels (Instagel I-III).

The glass plates were cleaned with absolute alcohol and three sides with spacers were sealed with 0.5 per cent agarose. After attaching the clamps to the sealed sides, 80 ml of Instagel containing 400 μl ammonium persulfate (APS) and 42 μl TEMED for polymerization was poured into horizontal plates tilted at an angle of 45° and a sharks tooth comb was inserted upside down. At least a 1-1.5 h was necessary for polymerization at rt. If the gel was kept o/n, the comb was covered with plastic wrap to prevent drying out. After polymerization the gel was set up right and the buffer chambers were filled with 1X TBE buffer. Before reinserting the comb, the gel surface was cleaned with syringe or a broken pasteur pipette to remove the urea.

Before loading the samples, the gel was pre-run for about an hour in 1X TBE buffer to heat the plates to 45-55°C in order to keep the DNA denatured. The electrophoresis conditions were 65-75 W, 35-45 mA and 1,800-2,000 V.

The samples were denaturated at 95°C for 5 min prior to loading and quenched on ice. One and a half to 3 μl from each sample were loaded onto gels and electrophoresed until the BPB reached the bottom of the gel in 6 per cent polyacrylamide gels and when the XC reached the bottom of the gel in 8 per cent polyacrylamide gels.

After electrophoresis, the glass plates were separated with spatula, the gel was transferred onto a 3MM Whatman paper and covered with plastic wrap. The gel was dried in a 80°C oven for 1 h and exposed to an X-ray film using an intensifying screen at -70°C for 1-16 h or without an intensifying screen at room temperature for o/n.

The X-ray film was developed manually for 2-3 min. It was rinsed with water two or three times and fixed for 4-10min. Then it was washed under tap water thoroughly.

D. Denaturing Gradient Gel Electrophoresis (DGGE)

Three regions of the FVIII gene were studied by DGGE: Exon 11, Exon 23, and Exon 24. A 40-nucleotide long GC-clamp was attached to the 5' end of one PCR primer of each pair of oligonucleotides (63). (See Appendix A.2)

1. Polymerase Chain Reaction (PCR)

All PCR reactions were performed in volumes of 50 μl. PCR conditions for each primer set were optimized by titrating Mg^{2+}, primer and dNTP concentrations.

For Exon 11 PCR, the reaction mixture contained 1X Promega Mg^{2+} free buffer, 2 mM $MgCl_2$, 200 μM each dNTP, 50 ρM of forward primer containing GC-clamp, 200 ρM of reverse primer and 2.5 units of Taq polymerase.

Exon 23 PCR reaction mixture contained 1X Promega Mg^{2+} free buffer, 5 mM $MgCl_2$, 600 μM each dNTP, 25 ρM each primer, and 2.5 units of Taq polymerase.

PCR reaction mixture for Exon 24 contained 1X Promega Mg^{2+} free buffer, 5mM $MgCl_2$, 800 μM each dNTP, 12.5 ρM of forward primer containing GC-clamp, 50 ρM of reverse primer, and 2.5 units of *Taq* polymerase. As usual, all samples were layered with two drops of mineral oil.

The annealing temperatures for the amplification of these three exons were chosen according to the melting temperature (T_m) of the primers.

After an initial denaturation at 94°C for 7 min, 35 cycles were carried out. Each cycle consisted of these following incubations: 1) at 95°C for 30 sec, 2) at 57°C for 30 sec (for Exon 11), at 60°C for 3 min (for Exon 23), at 52°C for 30 sec (for Exon 24), 3) at 72°C for 2 min (for exons 11 and 24) and at 60°C for 7 min

(for Exon 23). There was a final incubation at 72°C for 7 min at the end of 35 cycles for each exon.

5 μl aliquots were taken from the incubation mixtures and loaded with one μl 10X loading buffer A on a 1.8 per cent agarose gel for Exon 11 and 2 per cent agarose gel for Exon 23 and Exon 24 PCR product analysis. They were electrophoresed at 100-150 V and visualized under UV light.

For heteroduplex formation normal DNA samples were also amplified. Equal amounts of PCR products of both normal and patient DNAs for the same exon were mixed in a total volume of 20-40 μl. The mixtures were incubated for 5 min at 95°C for denaturation and for 20 min at 55°C for renaturation.

2. Preparation of the Denaturing Gradient Gels

The glass plates were cleaned with absolute alcohol and the side spacers (0.75 mm thick) were placed along the full length of the gel. The plates were inserted into two side frames containing several screws for tightening and placed on a base. After screwing, the plates were pressed again with two clamps located on both sides of the base. The vacuum grease was applied along the base. The comb was inserted between the plates at a 30-45° angle towards the left side (Figure IV.1).

The gradient maker had two chambers, 15-25 ml capacity per side, contacting with each other by a stopcock. The right chamber was connected to the peristaltic pump by another stopcock. The pump reached the gap between the glass plates through a thin plastic tube with a needle at its end.

Two solutions of equal volume (8 ml) were prepared from stock solutions to give the desired denaturant concentration range. A 4 ml solution without denaturant was also prepared. Then 80 μl APS and 3 μl TEMED were added to the solutions. The higher denaturant solution was poured into the right chamber of the gradient maker. This solution should be the first to exit the gradient maker and enter the plate cavity. Then the solution of lower denaturant concentration was poured into the left chamber. While stirring the solution of higher denaturant concentration by a magnetic stirrer, the stopcock between the two chambers and the stopcock entering the peristaltic pump were opened. The solutions were transferred entirely into the plate cavity and a 2-4 cm space was left under the comb. The stopcock between the chambers of the gradient maker was closed

and 4 ml gel solution without denaturant was poured into the right chamber. This solution reached the top of the plates to obtain smooth running conditions. The right side of the comb was inserted into the gel vertically.

At least 20-30 min was necessary for the polymerization. Polymerized gels can be kept at 4°C for a few days.

The denaturing gradient gels contained a concentration gradient of formamide and urea linearly increasing from the top to the bottom, parallel to the direction of electrophoresis (67).

3. Electrophoresis

A constant temperature must be maintained to ensure sharp, reproducible bands. Maintenance of an adequately uniform temperature in the gel required that the plates enclosing the gel be submerged in a well-stirred, temperature-controlled bath. A bath temperature of 60°C was chosen to somewhat exceed the melting temperature in the absence of denaturant of the most easily melted polydeoxyribonucleotide (67). So 1X TAE electrophoresis buffer was heated to 60°C in the aquarium (tank) prior to running.

The loading was done either before fixing the top (cathode) chamber by a pipette or after fixing and filling it with 1X TAE buffer by a syringe. The samples were loaded as 25-45 µl mixed with 5-10 µl of loading solution C prepared for the DGGE. Then the basement clamps were taken out and the gel with a full top chamber was placed into the tank. The peristaltic pump was connected to the apparatus so that buffer circulated from the aquarium into the top chamber (Figure IV.2). A pre-run with loading dye solution is helpful to see if the gel is properly set.

After electrophoresis, the glass plates were separated with a spatula, stained with EtBr for 5 min and visualised under UV.

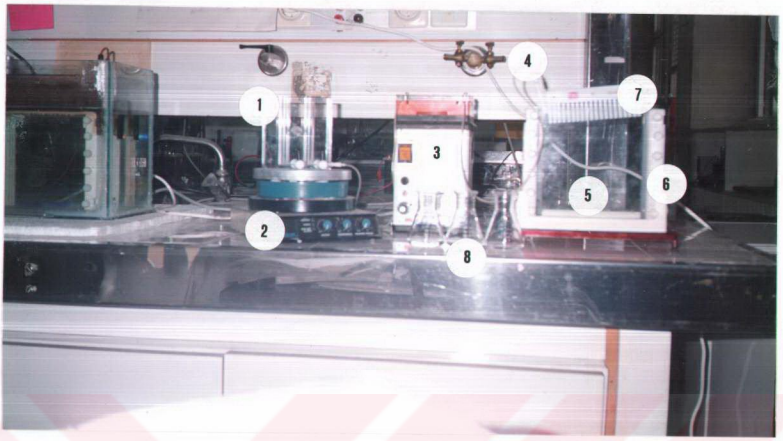

Figure IV.1. Denaturing gradient gel apparatus. 1. Gradient maker, 2. Magnetic stirrer, 3. Peristaltic pump, 4. Thin plastic tube with a needle at its end, 5. Glass plates, 6. Gel frame, 7. Comb, 8. Prepared gel solutions.

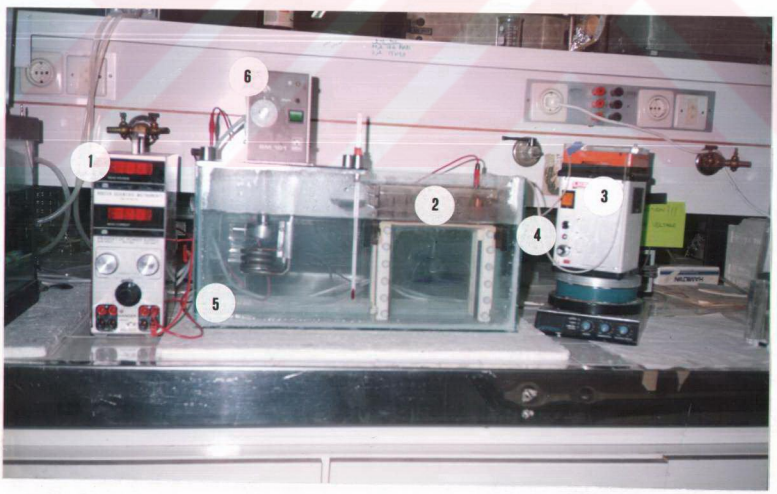

Figure IV.2. Denaturing gradient gel electrophoresis. 1. Power supply, 2. Cathode chamber, 3. Peristaltic pump, 4. Thin plastic tube, 5. Aquarium (tank), 6. Heater and stirrer.

E. DNA Sequencing with CircumVent™ Sequencing Kit

CircumVent™ Thermal Cycle Dideoxy DNA Sequencing protocols are based upon the dideoxynucleotide chain termination method of Sanger et al. (76). In the reaction, a suitable primer was annealed to a complementary DNA. This complex was incubated with the exonuclease deficient DNA polymerase in the presence of one of the four ddNTPs, generated four different sets of fragments, each set corresponding to terminations at specific nucleotide residues. In order to visualise the dideoxy terminated chains on the gel, ^{35}S was used for incorporation into the nascent chain by using α-[^{35}S] dATP.

One patient DNA, that presumably had a different base composition in the Exon 11 as judged by DGGE analysis, was sequenced using this kit.

1. Purification of the PCR Product

First Exon 11 of the FVIII gene was amplified according to the PCR conditions described before. Then to remove excess primers, dNTPs, Taq polymerase and unspecific PCR fragments, the PCR product was either applied to a Centricon-100 microconcentrator (Amicon) or eluted from a 1 per cent agarose gel by cutting a thin slice of the gel containing the band of interest.

The PCR product in the Centricon-100 was washed twice with two ml of distilled water by spinning it at 1,000xg for 25 min, the water was discarded and the filter was inverted and spun at 1,000xg for 2 min to transfer the product DNA into the upper cup. The amount of PCR product was tested on agarose gels.

In the other method, 300 µl of TE buffer was added to the sliced piece of the gel. It was freezed at -20°C and thawed at 37°C. This step was repeated and it was incubated at 37°C for o/n. Then the gel piece was thrown, the DNA was precipitated and dissolved in 5-15 µl of sterile distilled water.

2. Sequencing Reactions

Four microcentrifuge tubes were labelled as A, C, G, T and 3 μl of A, C, G, and T mixtures were added to these tubes, respectively. 2-10 μl of purified DNA (approximately 1 μg of DNA) was used as template in the reaction. The Exon 11 amplified DNA, 1.5 ρM of the primer 274 (see Appendix A.2), 1X CircumVent™ sequencing buffer, 1 μl 30X Triton X-100 solution, 2 units of $Vent_R$ (exo-) DNA polymerase and α-[^{35}S]dATP (500-1,200 Ci/mmol) were mixed in a microcentrifuge tube and the total volume was made up to 15 μl with sterile distilled water. The solution was mixed by gentle pipetting. 3.2 μl of this mixture was immediatly distributed to each of the four deoxy/dideoxy tubes. Each tube was layered with one drop of mineral oil and then placed in the thermal cycler for amplification.

The program consisted of 25 cycles. Each cycle was as follows: at 95°C for 20 sec, at 37°C for 20 sec, and at 74°C for 60 sec.

After the amplification, 4 μl of stop/loading buffer was added to each tube to terminate the reactions. Tubes would be stored at -20°C, for almost one week.

3. Electrophoresis and Autoradiography

The sequencing mixtures were run on 0.4 mm thick 6-8 per cent polyacrylamide gels. The preparation of these gels was the same with those used for Int13 $(CA)_n$ repeat analysis.

Typical conditions were 70-80 W, 35-45 mA and 1,600-1,900 V. Three μl from each termination tube was applied in each slot in the order of A, C, G, and T. The gel was run until the desired separation was achieved. BPB and XC included in stop/loading buffer were used as an indicator for the region of interest.

After drying in an oven at 80°C for 1 h, the gel was exposed to an X-ray film for one to seven days and developed manually as described before.

V. RESULTS

A. Carrier Determination and Prenatal Diagnosis by DNA Linkage Analysis

Five families that requested carrier determination and/or prenatal diagnosis were studied using the already established markers for linkage analysis in hemophilia A in the Turkish population (51). These markers were the two intragenic RFLPs *Hind*III (Intron 19), *AlwN*I (Intron 7) and one highly informative extragenic marker St14 (DXS52). The genotypes of individuals with respect to the intragenic markers were determined according to the restriction digestion patterns on the gels after amplification of the loci. The presence of the variable restriction site was designated by (+) genotype and its absence by (-) genotype. Homozygous males have only two genotypes designated as (+) or (-). Three possible genotypes (+/+), (-/-), or (+/-) exist for females. The *Hind*III PCR product contain an invariant site that acts as an internal control of amplification conditions.

The multiallelic St14 VNTR locus was examined directly by sizing of the PCR products. To avoid errors in determining allele sizes, which may be different from each other as small as 30 bp, it was necessary to include all family members on the same gel.

Family 48:

The mother (II.1) was considered a possible carrier because she had one affected son (III.1) and no other affected relatives (Figure V.1a). She (II.1, Lane 4) was informative for *Hind*III and St14 loci (Figure V.1b). The patient (III.1, Lane 3) received (-) *Hind* III allele and 700 bp St14 allele from his mother which showed that the disease segregated with this haplotype in the family. The daughter (III.2, Lane 5) was a possible carrier since she received the haplotype of the affected son. One of the mother's sisters (II.3, Lane 7) and her daughter (III.3, Lane 6) were also possible carriers since they received the same haplotype. The other sister (II.5, Lane 8) was excluded from being a carrier because she received the 1,300 bp St14 and (+) *Hind* III alleles not segregating with the disease from her mother. The diagnosis was based on the assumption that the grandmother (I.1) had the following genotype (-/-) for *Hind* III and

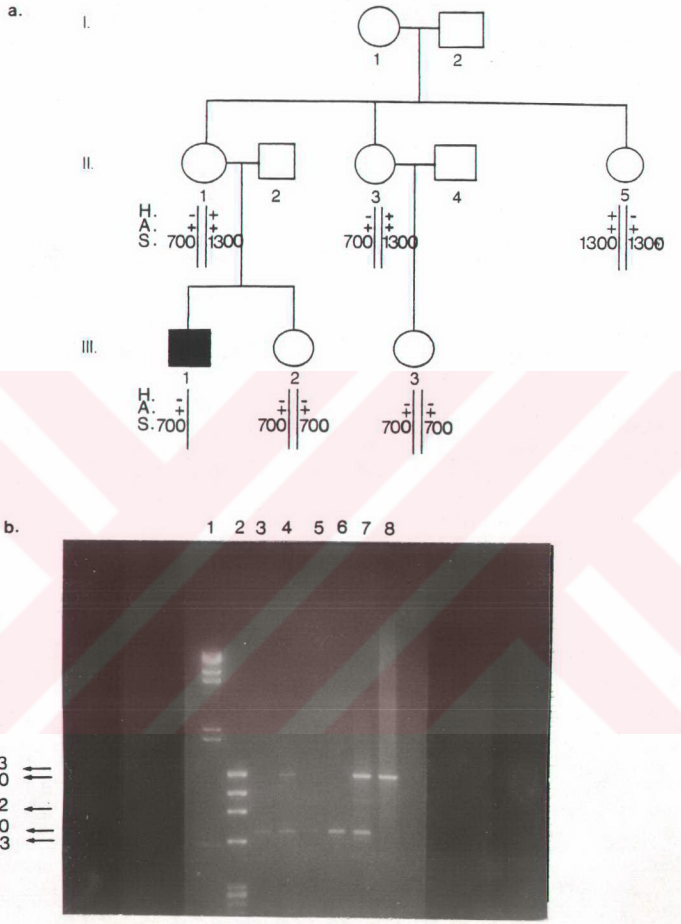

Figure V.1. Family 48

Pedigree of the family and the polymorphic marker genotypes of the members. H, A, and S denote *Hind*III, *AlwN*I, and St14, polymorphisms, respectively (a). The St14 genotypes of the family members are seen on a 1 per cent agarose gel. λ/*Hind*III and ϕ-x174/HaeIII DNA size markers are used in lanes 1 and 2, respectively. Lanes 3-8 include the family members III.1, II.1, III.2, III.3, II.3, and II.5, respectively (b).

1,300/700 bp for St14 and grandfather (I.2) had (+) *Hind* III, 1,300 bp St14. Because St14 is an extragenic marker, 5 per cent recombination risk exists in exclusion analysis of II.5.

Family 57:

The mother (II.3), in the first trimester of her first pregnancy, was at risk of being a carrier since she had an affected nephew (III.2) (Figure V.2a) and two brothers that died from an unknown cause in early infancy. The females (I.1, II.1, and II.3) were not informative for *Hind*III and *AlwN*I RFLPs. Therefore, St14 locus was used as the linkage marker. Her sister (II.1, Lane 5) carried the 1,300 and 1,390 bp alleles of St14 locus and her affected son (III.2, Lane 7) received the 1,300 bp (Figure V.2b). However, one of the normal son (III.1, Lane 6) also received this allele. This indicated that a recombination has occurred in II.1 between the mutant site and the polymorphic marker or it occurred sporadically in III.2. The mother (II.3, Lane 4) was excluded from being a carrier as she didn't receive 1,300 bp allele from I.1 and Chorionic Villus Sampling (CVS) was not performed on the fetus.

Family 59:

The mother (II.3) who requested prenatal diagnosis was considered a possible carrier since she had affected brothers and nephews (Figure V.3a). Her sister (II.1, Lane 4) was only informative for St14 locus (Figure V.3b). She had 1,570 bp and 2,400 bp alleles and her affected son (III.1, Lane 5) had 1,570 bp showing that the disease segregated with 1,570 bp allele (Figure V.3b). The mother (II.3, Lane 6) was informed that she had a 5 per cent risk of being a carrier because she didn't have this allele and CVS was not needed.

Family 77:

The mother (I.3) was a possible carrier with an affected son (II.1) and no other affected relatives (Figure V.4a). I.3 (Lane 2) was informative for *Hind*III polymorphism (Figure V.4b). The affected son (II.1, Lane 4) received (+) allele indicating that the disease segregated with the (+) allele. The pregnant daugther (II.3, Lane 3) was a possible carrier since she received the (+) *Hind* III allele from her mother (I.3). In this case, CVS was necessary. The sex of fetus (III.1, Lane 5) was determined as female since she was heterozygous for a FIX polymorphism (data not shown) and her *Hind* III genotype was (-/-). Therefore, the fetus was diagnosed as healthy.

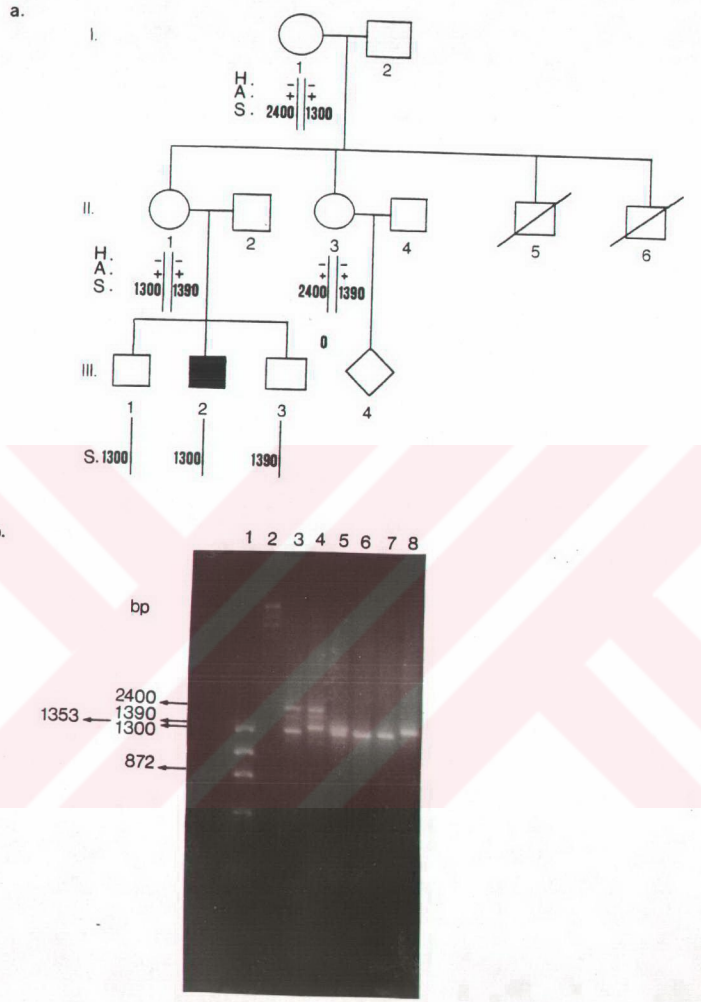

Figure V.2. Family57

Pedigree of the family and the polymorphic marker genotypes of the members. H, A, and S denote *Hind*III, *AlwN*I, and St14 polymorphisms, respectively (a). The St14 genotypes of the family members are seen on a 1 per cent agarose gel. ϕ-x174/*Hae*III and λ/*Hind*III size markers are used in lanes 1 and 2, respectively. Lanes 3-8 include the family members I.1, II.3, II.1, III.1, III.2, and III.3, respectively (b).

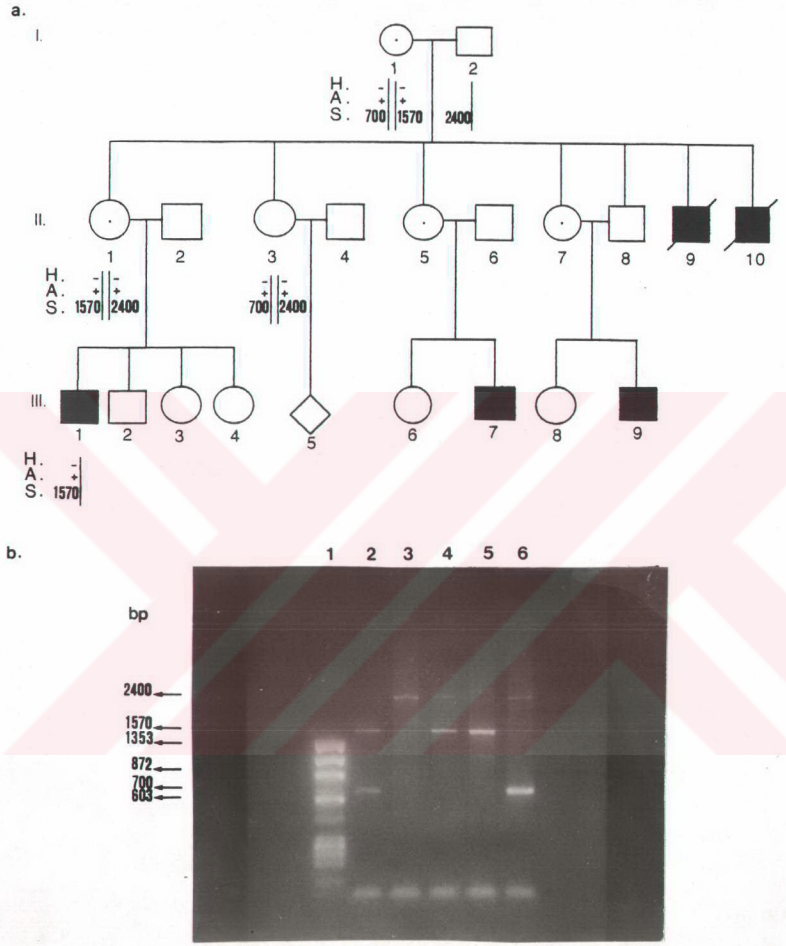

Figure V.3 Family 59

Pedigree of the family and the polymorphic marker genotypes of the members. H, A, and S denote *Hind*III, *AlwN*I, and St14 polymorphisms, respectively (a). The St14 genotypes of the family members are seen on a 1 per cent agarose gel. ϕ-x174/*Hae*III DNA size marker is seen in lane 1. Lanes 2-6 include the family members I.1, I.2, II.1, III.1, and II.3, respectively (b).

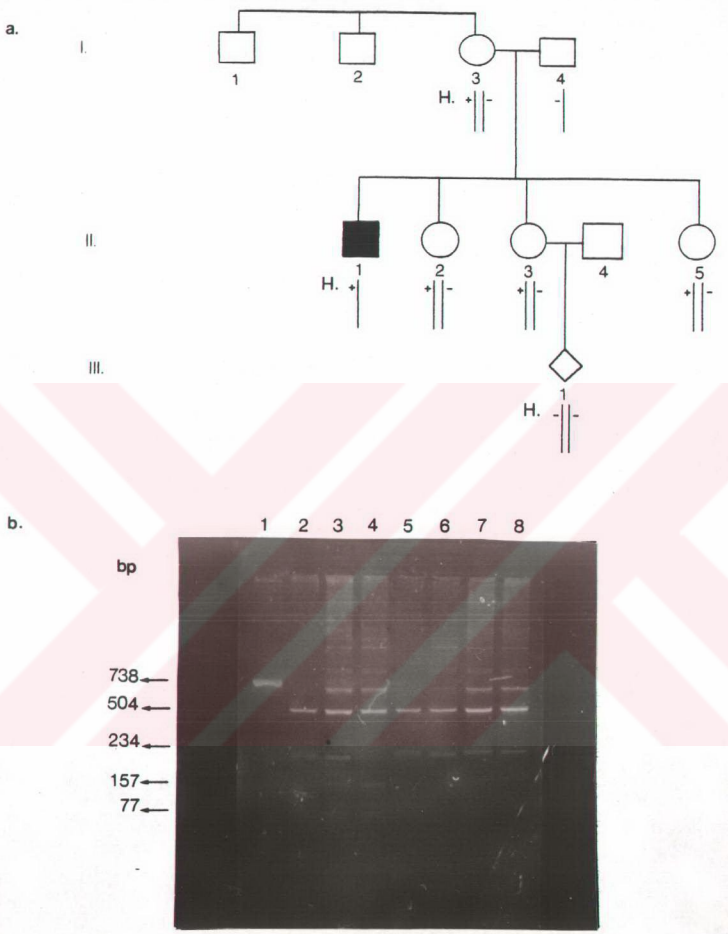

Figure V.4. Family 77

Pedigree of the family and the *Hind*III polymorphic marker genotype of the family members (a). The*Hind*III digestion patterns of the family members are seen on a 1.8 per cent agarose gel. Lane 1 shows *Hind*III amplification product used as a size marker. Lanes 2-8 refer to the family members I.3, II.3, II.1, III.1, I.4, II.2, and II.5 respectively (b).

Family 96:

The mother (I.1) was a possible carrier with an affected son (II.1) and she requested prenatal diagnosis (Figure V.5a). *Hind*III polymorphism showed that the disease segregated with (+) allele (Figure V.5b). The father (I.2, Lane 5) carried the (-)*Hind* III allele. The *Hind* III genotype of the fetus (II.2, Lane 4) was (+). Therefore the fetus was at risk of being an affected male.

B. DNA Linkage Analysis Using the Hypervariable $(CA)_n$ Repeat in Intron 13

Intron 13 $(CA)_n$ repeat provides the most highly informative multi-allelic intragenic marker so far available for FVIII gene tracking studies in hemophilia A kindreds (44). In the frame work of this thesis we have carried out preliminary studies to establish the use of Intron 13 $(CA)_n$ repeat polymorphism in linkage analysis.

As it is explained in the methods section the DNA fragment involving the $(CA)_n$ repeat is amplified by PCR using a radioactive primer and subjected to electrophoresis on sequencing gels that are able to distinguish fragments that differ in length by one basepair. Eight alleles known to exist in Caucasians that differ by 2 bp, the shortest allele with $(CA)_{16}$ repeat is 133 bp in length. A known length of DNA fragment (260 bp) was also end-labeled by PCR and electrophoresed along with the PCR product of the $(CA)_n$ repeat to be able to locate the $(CA)_n$ repeat on the gels. It showed that 3h electrophoresis was enough to see the $(CA)_n$ repeat alleles on the sequencing gels (data not shown). Instead of using agarose gels for the quantitative analysis of the radioactively labeled PCR products, dilutions (1X, 1/10X, 1/20X, 1/50X) of the PCR products were prepared and applied on sequencing gels in duplicates (Figure V.6). It was observed that 1/10 dilution of the PCR product and o/n exposure of the autoradiogram were optimal to visualize the alleles.

Seven families were chosen and analyzed for Intron 13 dinucleotide repeat polymorphism. The results of the two families are given in detail. As in the case of St14 VNTR, this multi-allelic dinucleotide repeat polymorphism is only useful when key family members are included into the DNA linkage analysis.

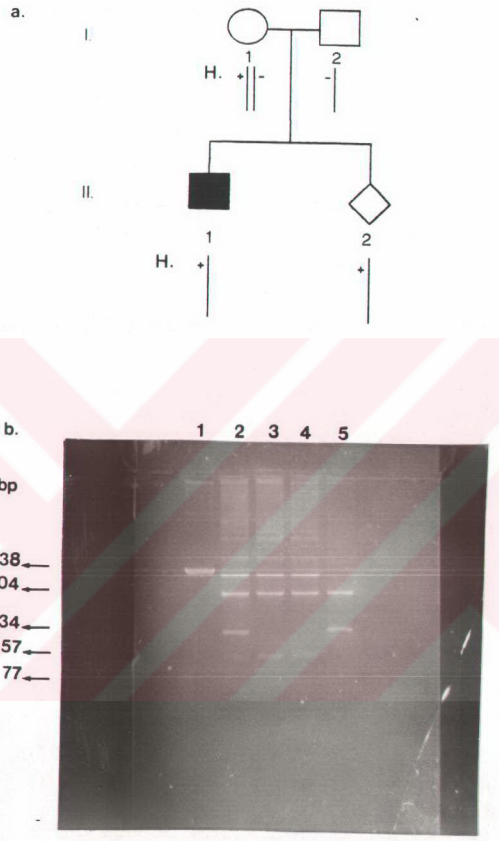

Figure V.5. Family 96

Pedigree of the family and the *Hind*III genotypes of the family members (a). The *Hind*III digestion patterns of the family members are seen on a 1.8 per cent agarose gel. Lane 1 shows *Hind*III amplification product used as a size marker. Lanes 2-5 refer to the family members I.1, II.1, II.2, and I.2, respectively (b).

Figure V.6 Optimization of the amount of $(CA)_n$ repeat PCR product. Groups 1-4 refer to the following dilutions of the same PCR products: 1X, 1/10X, 1/20X and 1/50X, respectively.

Family 48:

Figure V.7a shows the pedigree of this family and Figure V.7b shows $(CA)_n$ repeat alleles of the family members on sequencing gels. The mother (II.1, Lane 2) was heterozygous and her alleles were designated as 1/2. Her affected son (III.1, Lane 1) received allele 1 indicating that the disease segregated with allele 1. His sister (III.2, Lane 3) was a possible carrier since she received allele 1 from her mother and allele 3 from her father. One of the mother's sisters (II.3, Lane 4) was a possible carrier with 1/2 genotype and her daughter (III.3, Lane 6) was also a possible carrier since she is homozygous for allele 1. The other sister (II.5, Lane 5) was not a carrier with 2/3 genotype. These results confirmed the previous analysis that used *Hind*III and St14 markers (Figure V.1). $(CA)_n$ repeat analysis in this family enabled us to predict the alleles of the grandparents I.1 and I.2 that were 1/3 and 2, respectively.

Family 77:

Figure V.8a shows the pedigree of this family and Figure V.8b shows $(CA)_n$ repeat alleles of the family members on sequencing gels. The patient's mother (I.3, Lane 4) had 1/2 genotype and the patient (II.1, Lane 1) received allele 2 indicating that the disease segregated with allele 2. The father (I.4, Lane 3) carried allele 1. The sister (II.2, Lane 2) has the genotype 2/1 and is a possible carrier. Although the mother's $(CA)_n$ genotype (II.3, Lane 6) is not seen on the gel, the fetus (III.1, Lane 7) was diagnosed as healthy female since she was homozygous for allele 1 and also according to previous analysis by *Hind*III polymorphic marker. The father of the fetus (I.4, Lane 3) is also shown to have allele 1. The genotype of I.1 (Lane 5) is different than the others in the family and is designated as allele 3. His brother (I.2, Lane 8) has allele 1 suggesting that their mother was heterozygous carrying alleles 3/1. The mother of the patient (I.3) must have received allele 2 that segregates with the disease from his father. Since the disease is sporadically seen in II.1, we can assure that the mutation has originated either in the germ cell of the granfather or the mother of the patient.

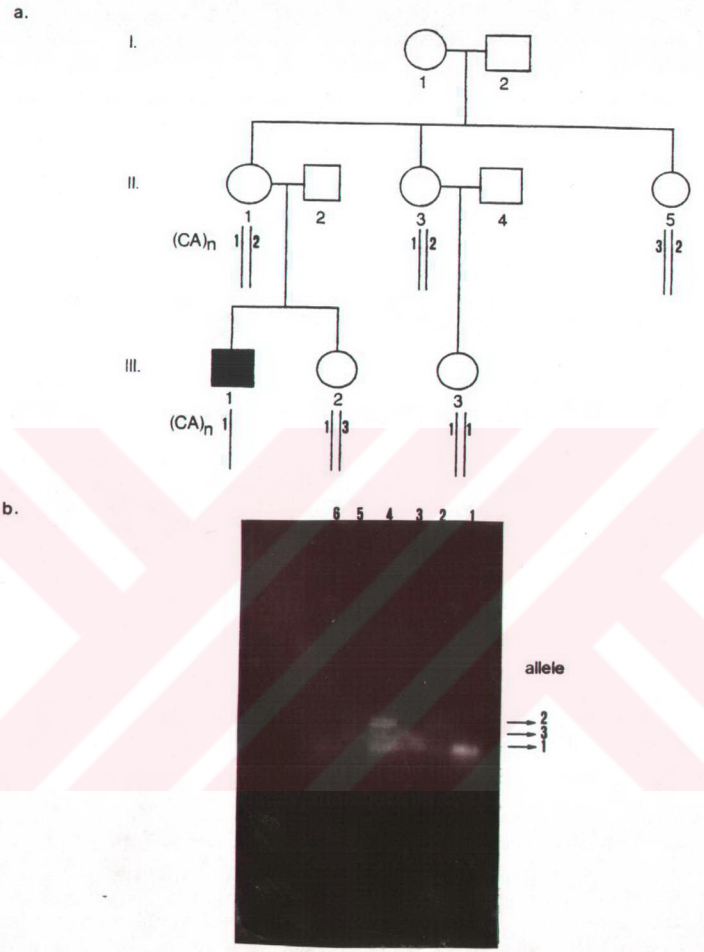

Figure V.7. The analysis of Intron 13 $(CA)_n$ repeat polymorphism of some members of the family 48. The genotypes of the family members are given (a). The allelic pattern of the family members are seen on a 8 per cent sequencing gel and indicated by arrows (b). Lanes 1-6 include the family members III.1, II.1, III.2, II.3, II.5, and III.3, respectively.

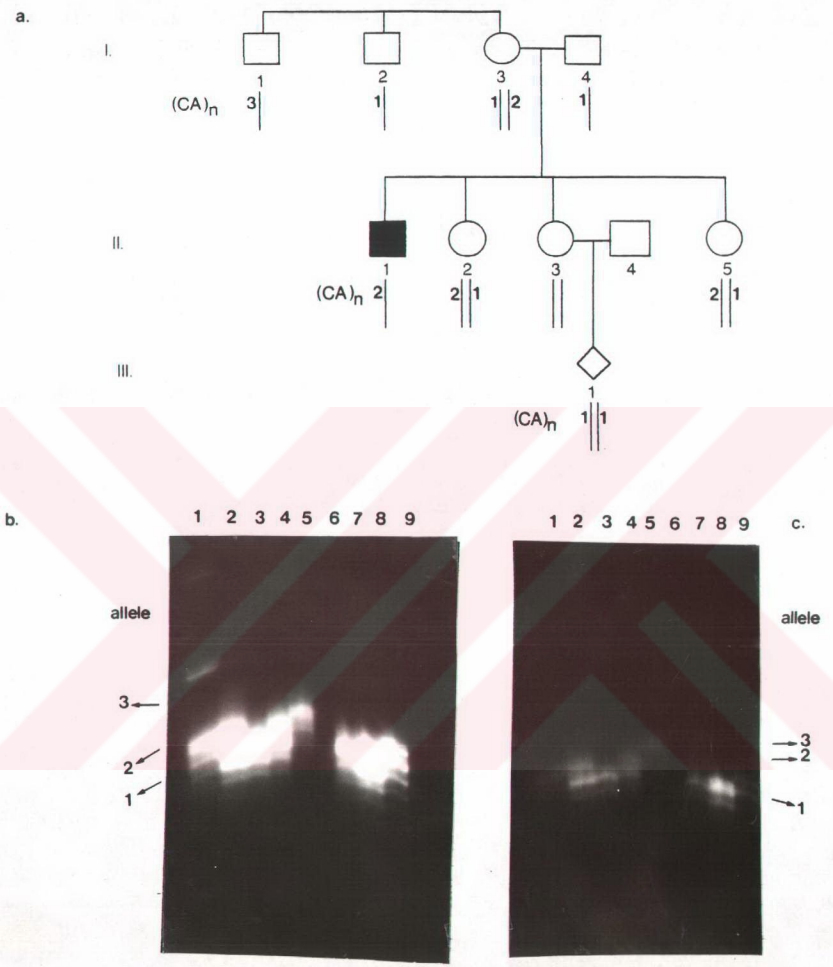

Figure V.8. The analysis of Intron 13 $(CA)_n$ repeat polymorphism of some members of the family 77 members. An o/n and a 3 h exposure of the X-ray film are shown in figures (b) and (c), respectively. The allelic pattern of the family members are seen on a 8 per cent sequencing gel. Lanes 1-9 represent the family members II.1, II.2, I.4, I.3, I.1, II.3, III.1, I.2, and II.5, respectively. The genotypes of the family members are given (a). The mother (II.3) genotype can be predicted from the other genotypes of the family members.

C. DGGE Analysis

Identifications of the causative mutations is the ultimate goal in the accurate determination of carriers and prenatal diagnosis and it is a challenge as to the understanding of the molecular pathogenesis of the disease.

In an attempt to identify point mutations in the FVIII, DGGE analysis of the gene was initiated. It is possible to screen the nine kb coding sequence of the FVIII gene and 50 splice junctions with approximately three primer sets. We have chosen three exons of the FVIII gene each of which can be amplified by a single pair of primers. These were Exon 11, Exon 23, and Exon 24.

1. Optimization of the PCR Conditions

The concentrations of the purchased primers were determined spectrophotometrically and on minigels (2 per cent NuSieve + 1 per cent agarose).

$MgCl_2$ is the cofactor of enzyme *Taq* polymerase. It is also bound by free dNTPs and primers in the reaction mixture. Therefore, Mg^{2+} titrations were carried out for each primer set. Figures V.9 and 10 show Mg^{2+} titration for Exon 11 and Exon 24 at a dNTP concentration of 200 μM. dNTP titration was also carried out for Exon 23 and Exon 24 to improve the amplification conditions.

All PCR reaction conditions optimized for each exon are given in Table V.1. The sizes of the amplified exons 11, 23 and 24 were 324 bp, 260 bp and 289 bp, respectively.

2. Determination of the Optimal Range of Denaturant Concentration and the Period of Time for Electrophoresis

In order to determine the optimal range of denaturants in the DGGE gel, the same PCR product was loaded in groups with one hour intervals onto a gel containing a gradient of 20-80 per cent. The electrophoresis was stopped after

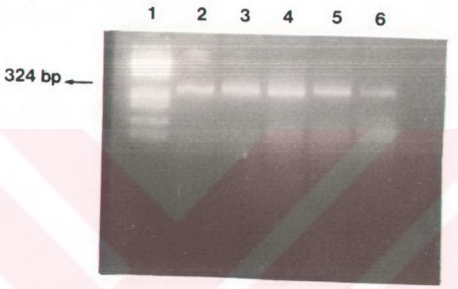

Figure V.9. The Mg^{2+} titration of the PCR reaction that amplify Exon 11. Lane 1 refers to ϕ-X174/HaeIII size marker. Lanes 2-6 refer to following Mg^{2+} concentrations 1 mM, 2 mM, 3 mM, 4 mM, and 5 mM. Arrow indicates the size of the amplified Exon 11.

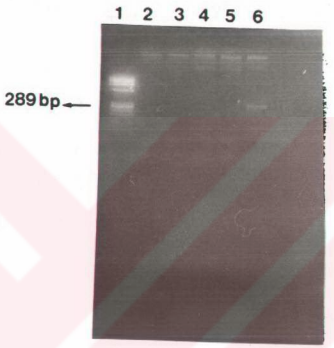

Figure V.10. The Mg^{2+} titration for Exon 24 PCR. Lane 1 shows the ϕ–X174 DNA size marker. Lanes 2-6 refer to 1 mM, 2 mM, 3 mM, 4 mM, and 5 mM $[Mg^{2+}]$ respectively. The arrow shows the amplified product of Exon 24.

five to six hours from the initial loading at 0 h. The position of the gel corresponding to 20 per cent denaturant and 80 per cent denaturant was marked and measured in "cm". Then this 60 per cent difference was divided to the measured length to find out the denaturant concentration per cm. The distance moved by the first group representing 0 h loading from 20 per cent denaturant position was measured and multiplied with the denaturant concentration per cm. The 20 per cent denaturant concentration was added to this product. The result was the optimum denaturant concentration for a specific PCR product. Generally a difference of 25-30 per cent between the top and the bottom of the gel is ideal (67). Our working conditions were between 10 per cent under and above the optimum denaturant concentration.

Figures V.11 and 12 show the application of this strategy for exons 23 and 24, respectively. Calculations are given below:

For Exon 23:

60% : 13 cm = 4.6%/cm
2.9 cm (1st group stopped) x 4.6%/cm = 13.34%
~13.5% + 20% = ~34%

The optimum denaturant range for this fragment is 25-45 per cent.

For Exon 24:

60% : 13.6cm = 4.41%/cm
6.9 cm (1st group stopped) x 4.41%/cm = 30.42%
~30% + 20% = 50%

The optimum range is 40-70 per cent.

The travel time needed to reach leveling off between groups specifies the minimum adequate run time (67).

All denaturant concentration ranges and electrophoresis times used are given in Table V.1.

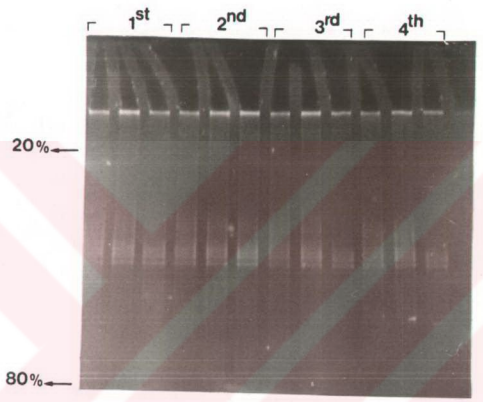

Figure V.11. Determination of the optimal range of denaturant concentration and running time for Exon 23 on DGGE gels. Four groups of same sample were loaded at 1 h intervals with a total run time of 6 h. Thus the samples at groups 1, 2, 3, and 4 run for 6 h, 5 h, 4 h, and 3 h, respectively.

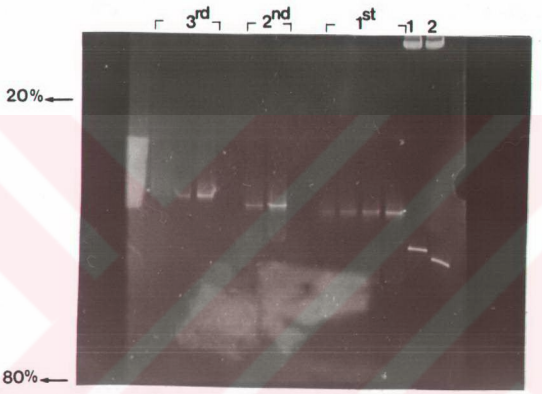

Figure V.12. Determination of the optimal range of denaturant concentration and running time for Exon 24 on DGGE gels. Three groups of same sample were loaded at 1 h intervals with a total run time of 5 h. The the samples at groups 1, 2, and 3 run for 5 h, 4 h, and 3 h, respectively. Lane 1 indicates one mutant plasmid DNA and lane 2 indicates its normal fragment both being used as control markers.

Table V.1. PCR conditions, optimal denaturant concentration range and electrophoresis time for each exon. F.P. : Forward Primer; R.P. : Reverse Primer.

Exons	$[Mg^{2+}]$ (mM)	[dNTP] (µM)	[F.P.] pM	[R.P.] pM	Taq pol. (units)	PCR cycle conditions	Fragment size (bp)	Denaturant concentration range	Electrophoresis time (h)
Exon 11	2	200	50	200	2.5	95°C, 30" 57°C, 30" 35 cycles 72°C, 2'	324	40-70%	5.5-6
Exon 23	5	600	25	25	2.5	95°C, 30" 60°C, 3' 35 cycles 60°C, 7'	260	25-45%	6-6.5
Exon 24	5	800	12.5	50	2.5	95°C, 30" 52°C, 30" 35 cycles 72°C, 2'	289	40-70%	5.5-6

3. Screening for Mutations in Three Exons of the FVIII Gene of Hemophilia A Patients

After optimization of the DGGE conditions, 78 hemophilia A patients of Turkish origin have been screened for base composition differences in Exon 11, Exon 23 and Exon 24.

We observed a single change in the migration pattern of 78 patients in Exon 11 (Figure V.13, Lane 5) and two samples migrated differently in Exon 23 (Figure V.14, lanes 10 and 12) in DGGE analysis suggesting that they have different melting properties, i.e., different base composition. There was not a single sample out of 78 patient DNA that migrated at a different position for Exon 24.

The heteroduplex formation is another technique that makes the DGGE system much more sensitive. This method depends on forming heteroduplex between one strand of the probable mutant and a complementary strand from the wild-type sequence. In the heteroduplex formation four duplexes can form: normal homoduplex, mutant homoduplex, normal-mutant heteroduplex, and mutant-normal heteroduplex. Each fragment exhibits a different position in the denaturing gel. Therefore, each patient sample was mixed with a positive control amplified DNA and examined for heteroduplex formations for each exon.

Figures V.15 and 16 show heteroduplex analysis of Exon 11 and Exon 23 samples on the denaturing gradient gels, respectively. The gels were prepared and ran at identical conditions with previous exon analysis. In heteroduplexed molecules, i.e., DNA molecules in which one strand is derived from a normal control and one strand is derived from a hemophilic sample - mismatching or a loop structure due to a deletion or insertion destabilize the DNA, causing a significant upward shift on the denaturing gradient gel. The original homoduplexed bands are also seen in the figures. The heteroduplex formations confirmed the existence of a different base composition in three patients, one in Exon 11 and two in Exon 23.

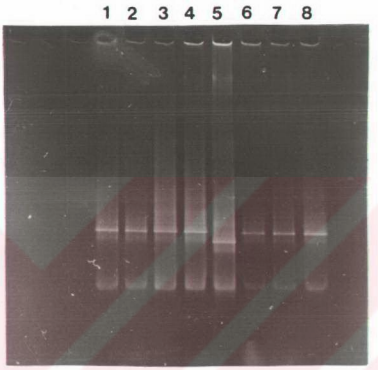

Figure V.13. DGGE analysis of Exon 11. Eight different patients' DNA amplified for Exon 11 are seen on the gel. All showed the same pattern except the DNA in Line 5 (61HA248). .

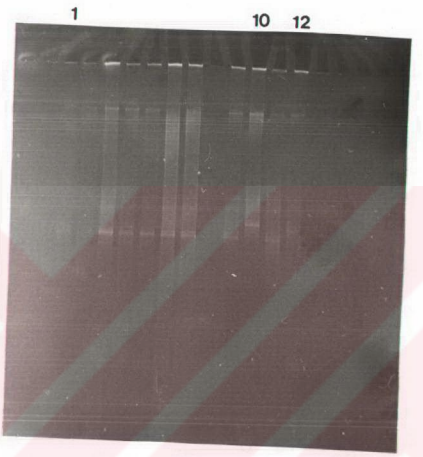

Figure V.14. DGGE analysis of Exon 23. The patients' DNA in Lanes 1((55HA211) and 12 (67HA279) show a different pattern.

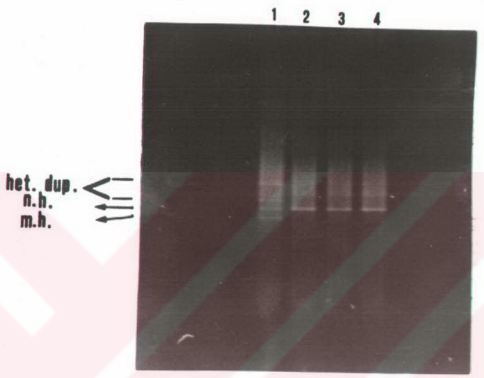

Figure V.15. The heteroduplex formation for Exon 11 DGGE analysis. Lane 1 shows the heteroduplex formation between the patient's DNA (61HA248) and a normal DNA. The arrows indicate the heteroduplexes, normal and mutant homoduplexes. Lanes 2-4 represent three patients' DNAs mixed with the same normal DNA. Heteroduplexes were not observed in these samples.

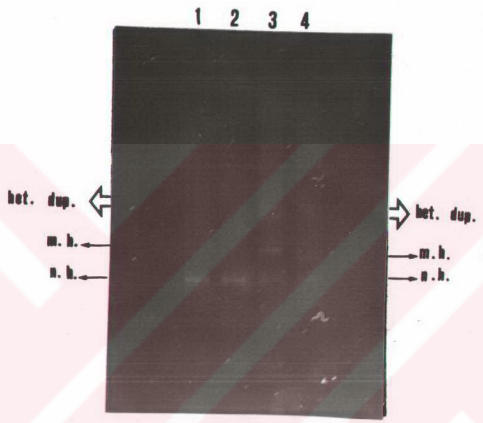

Figure V.16. The heteroduplex formation for Exon 23 DGGE analysis. Lanes 3 and 4 show the heteroduplex formation between the patients' DNAs (55HA211 and 67HA279, respectively) and a normal DNA. The arrows indicate the heteroduplexes, normal and mutant homoduplexes. Lanes 1-2 represent two patients' DNAs mixed with the same normal DNA. Heteroduplexes were not observed in these samples.

D. DNA Sequencing

In an attempt to locate the change in base composition of Exon 11 that had a different migration pattern on the DGGE gel of one patient, DNA sequence analysis was carried out by using CircumVent™ Thermal Cycle Dideoxy DNA Sequencing Kit.

Figure V.17 shows the sequencing pattern of Exon 11 of the patient and a normal individual. Approximately, 200 bp sequences were read, but not a single base difference was observed between the patient and the normal individual. The sequence analysis did not cover the complete amplified sequence of 324 bp since one of the PCR primers (without GC-clamp) was used as the sequencing primer and additional primers are needed to read the complete 324 bp sequence.

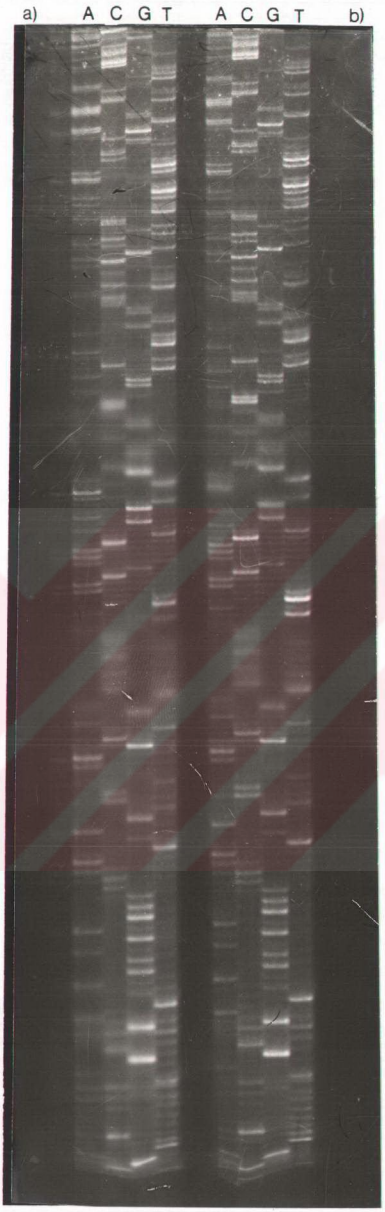

Figure V.17 The sequencing gel of Exon 11 from patient 61HA248 (a) and and a normal individual (b). The figure shows the comparison of approximately 200 bp of Exon 11 sequence.

VI. DISCUSSION

The work presented in this thesis involves the preliminary analysis of an intragenic VNTR site that can be used in DNA linkage analysis of hemophilia A afflicted families and establishes the use of DGGE as a screening method for the identification of Hemophilia A mutations, in the Turkish population.

Because of the large size of the FVIII gene and its heterogenous point mutations, the linkage analysis is still the major method of choice for rapid carrier identification and prenatal diagnosis. The strategy for linkage analysis for any particular family must take into account the factors including the site of the polymorphism, the degree of heterozygosity of the marker, and the ethnic origin of the family (20). The recently described hypervariable CA tandem repeats polymorphism in Intron 13 of the FVIII gene (44) appears to be informative in approximately 80 per cent of females and thus represents the most appropriate marker for carrier identification and prenatal diagnosis. In order to establish the routine use of this marker in our linkage studies we determined the conditions necessary for each PCR amplification of the locus and analysis of genotypes on sequencing gels.

$(CA)_n$ repeat alleles differ by a length of two basepair. However, the alleles do not appear as sharp bands on the gels due to the polymerase slippage, instead each allele is represented by several bands, the longest and the sharpest being the actual allele.The accurate length of alleles can be determined by sequencing. However, family studies are possible by comparing the genotypes of family members on the same gel without actually knowing the allele size. We were able to show the agreement of Intron 13 $(CA)_n$ repeat genotypes to previous linkage analysis studies in two families. Since multi-allelic polymorphisms are extremely useful in haplotype analysis and discriminating independent mutations, the number and size of Intron 13 $(CA)_n$ repeat polymorphic alleles should be determined for the Turkish population. This will also reveal whether allelic variation exists between the Turkish population and others. It is possible to introduce DIG-labeling to the study of this polymorphic site which will eliminate the use of radioactivity and enable its routine use as a linkage marker.

Gross gene rearrangements, such as deletions, insertions, and duplications, account for only five per cent of the molecular defects in hemophilia A patients, while point mutations account for the remaining 95 per cent of defects, nearly all of which have been found in exons (77). Detection of all

possible point mutations in FVIII gene is difficult because of its large size, and the high frequency of the *de novo* mutations. Over 80 different point mutations have been found in the FVIII gene by a combination of Southern blotting, oligonucleotide discriminant hybridization, Denaturing Gradient Gel Electrophoresis (DGGE), chemical cleavage, and DNA sequencing (70).

The majority of point mutations so far detected are located in exons 8, 11, 14, 18, 23, 24, and 26. This is due to the fact that exons 14 and 26 comprise 55 per cent of the length of the FVIII mRNA and to the presence of CGA (Arg) codons within readily screened *Taq* I restriction sites in exons 18, 23, 24, and 26. The short exons 7, 11, 12, and 16 appear to harbour a disproportionate number of mutations (63, 64). In spite of great effort, point mutations in severe hemophiliacs has not been identified. It is assumed that the mutations causing severe hemophilia A may reside in introns (especially Intron 22) which has not been screened yet.

DGGE of amplified PCR products with a 40-nt GC-clamp is a powerful tool for mutation-screening of large genes. For this reason we initiated DGGE analysis of the FVIII gene in Turkish patients. We have chosen exons 11, 23, and 24 to screen since they are relatively short fragments so they can be amplified by only one PCR primer set and relatively frequent mutations have previously been found in these exons. We have used the PCR primers that were chosen after computer analysis to assure that the regions of interest reside within the lowest melting domains (63). After setting up the PCR conditions, we determined the optimal DGGE conditions for each exon by varying denaturant concentrations and time of electrophoresis to maximize the difference in mobility between homoduplexes of normal and mutant DNA.

We established the conditions to use DGGE method to detect possible sequence differences in the coding regions of the FVIII gene and we screened 10 per cent of the FVIII gene for possible point mutations by analyzing the DNA of 78 Turkish patients for these three exons

Only one patient in Exon 11 and two different patients in Exon 23 showed different migration patterns. In Exon 24 we couldn't detect any difference in the migration pattern of 78 patients. In spite of the large patient sample, the observation of only three possible mutations indicated the heterogeneity of regions carrying a mutation.

Finally, we combined PCR products from normal individuals and patients to observe heteroduplexes since mutations can be detected by heteroduplexes even when mutant DNA is not distinguishable from normal DNA after DGGE.

Direct DNA sequencing of one patient showing a different pattern in the Exon 11 DGGE analysis were carried out. We couldn't see any sequence

difference within the 200 bp sequence read. Since the reverse PCR primer was used as the sequencing primer and α-[^{35}S] dATP incorporation was used to label the sequencing reaction products, it was not possible to read the initial sequences of the amplified exon at the 3' end. In addition, the amplified product of the exon is 324 bp in length which is above the range of length that can be sequenced by one primer.

Identification of mutations causing genetic disorders serve many functions. They may reveal new mechanisms of mutations, or genetic rearrangements and information about the organization of the genes; provide information about genotype/phenotype relationships and accurate means of carrier detection and prenatal diagnosis. Direct sequencing of the FVIII gene, is practically not possible to detect the causative mutation. Therefore, we conclude that the use of DGGE screening method in detecting hemophilia A mutations is feasible and worthwhile. It will even be more worthwile to apply this technique to screen for mutations in intronic sequences as more sequence data are being available.

APPENDIX A

1. PCR Primer Sequences of the Factor VIII Polymorphisms

MARKER	PRIMER SEQUENCE
Hind III	F 5' AAGGTCCTCGAGGGCGAGCATCTACATGCTGGGATGAGC
	R 5' AAGGTCGGATCCGTCCAGAAGCCATTCCCAGGGGAGTCT
AlwN I	F 5' TAATGTACCCAAGTTTTAGG
	R 5' TATAGAACAGCCTAATATAGCAAGACACTC
St 14	F 5' GGCATGTCATCACTTCTCTCATGTT
	R 5' CACCACTGCCCTCACGTCACTT
Int.-13 $(CA)_n$	F 5' TGCATCACTGTACATATGTATCTT
	R 5' CCAAATTACATATGAATAAGCC

F - Forward Primer
R - Reverse Primer

2. PCR Primer Sequences for DGGE Analysis

EXON	PRIMER SEQUENCE
Exon 11	F 5' CGCCCGCCCCGCCCGCCGCCCGCCCCGCCCGCCGC CCGCATGGTTTTGCTTGTGGGTAG R 5' GGATCCGACATACACTGAGAATGAA
Exon 23	F 5' CGCCCGCCCCGCCCGCCGCCCGCCCCGCCCGCCGCC CGCCTCTGTATTCACTTTCCATG R 5' AAGGATATGGGATGACTTGGCACT
Exon 24	F 5' CGCCCGCCCCGCCCGCCGCCCGCCCCGCCCGCCGCC CGCGCTCAGTATAACTGAGGCTG R 5' CTCTGAGTCAGTTAAACAGT

F - Forward Primer
R - Reverse Primer

APPENDIX B

1. DNA Size Markers

a. Lambda DNA *Hind* III Digest

FRAGMENT	BASEPAIR
1	23,130
2	9,416
3	6,557
4	4,361
5	2,322
6	2,027
7	564
8	125

b. ϕ-X174 DNA - *Hae* III Digest

FRAGMENT	BASEPAIR
1	1,353
2	1,078
3	872
4	603
5	310
6a	281
6b	271
7	234
8	194
9	118
10	72

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