MOLECULAR ANALYSIS OF THE FACTOR IX GENE

IN THE TURKISH POPULATION

by

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ABSTRACT

Hemophilia B is known as the third common hereditary coagulopathy. This X-linked recessive disease is caused by the production of either reduced amounts or functionally defective forms of the coagulation factor IX. Hemophilia B is very heterogeneous at both phenotypic and genotypic levels. Gene therapy seems to promise a cure for the disease within the next decade, however, the current replacement therapy either carries the risk of viral transmission and/or is an economic burden. The number of hemophilia B patients in our country is expected to be about 1000. In an attempt to undertake a comprehensive molecular analysis of the FIX gene in the Turkish population 41 hemophilia B patients were screened for mutations by ddF and direct DNA sequencing to contribute to the knowledge of genotype-phenotype correlations in hemophilia B and to construct a Turkish mutation profile. A hypervariable polymorphic site within the FIX gene have also been analyzed, which can be used as a tool in molecular evolution studies to observe genotypic variation within and among populations. Thirty-two mutations were identified in Turkish hemophilia B patients, including 4 novel single base changes and 2 novel gross rearrangements. The mutation profile of the Turkish population was found to be similar to the general profile observed worldwide, with point mutations making up over 90 per cent of causative mutations. Haplotype analysis revealed the independent origin of 4 recurrent mutations. All patient data was compiled in a national database, which efficiently shows the mutation/phenotype/haplotype relations. The analysis of the hypervariable region in intron 1 in 85 Turkish individuals revealed the presence of a novel allele and showed that the Turkish population carries the Caucasian specific allele as the most frequent one.

ÖZET

Hemofili B, X-e bağlı çekinik kalıtım gösteren ve üçüncü derecede sıklıkla gözlenen bir koagülopatidir. Faktör IX proteinin bozukluğu veya eksikliği sebebiyle oluşan hastalık, hem genotipik hem de fenotipik açıdan oldukça heterojendir. Hastalığın şu andaki tedavisi replasman terapisi ile yapılmaktadır. Bu yöntem hem pahalıdır, hem de kan yoluyla geçen hastalıkların bulaşma riskini taşımaktadır. Hastalığın kesin tedavisinin, gen terapisi ile mümkün olması beklenmektedir. Ülkemizdeki hemofili B hastalarının sayısı 1000 olarak tahmin edilmektedir. Faktör IX geninin moleküler analizinin Türk toplumunda detaylı bir şekilde yapılabilmesi amacı ile, 41 hemofili B hastası ddF ve doğrudan dizi analizi yöntemleri ile taranmıştır. Bu hastaların mutasyonlarının bulunması, hemofili B'deki genotip-fenotip ilişkisinin anlaşılması ve Türk toplumu için bir mutasyon profilinin çıkartılması açısından büyük önem taşımaktadır. Ayrıca, faktör IX geninin 1. intronunda bulunan ve moleküler evrim çalışmalarında toplum içi ve toplumlar arası genotipik farklılıkların incelenmesinde kullanılabilen çok değişken bir polimorfik bölge de incelenmiştir. Türk hemofili B hastalarında, 4 tane yeni tanımlanmış olan tek baz değişikliği ve 2 tane de yeni tanımlanmış büyük değişiklikler olmak üzere toplam 32 mutasyon belirlenmiştir. Türk toplumunun faktör IX mutasyon profilinin, tüm dünyada gözlenen mutasyon profili ile aynı olduğu bulunmuştur. Bu profilde, nokta mutasyonları hastalığa sebep olan tüm mutasyonların yüzde 90'ından fazlasını oluşturmaktadır. Hastaların haplotip analizleri sonucunda ise, şimdiye kadar saptanan 4 aynı mutasyonun her vakada birbirinden bağımsız olarak meydana geldiği anlaşılmıştır. Hastalara ait tüm veriler, mutasyon-fenotip-haplotip ilişkilerini etkin bir biçimde gösteren bir ulusal veri bankasında toplanmıştır. Faktör IX geninin 1. intronunda bulunan çok değişken polimorfik bölgenin 85 Türk bireyinde incelenmesi sonucunda, bir yeni alel bulunmuş ve Türk toplumunda beyaz ırka özgü alelin en sıklıkla görülen alel olduğu gözlenmiştir.

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I. INTRODUCTION

A. Blood Coagulation

Blood coagulation, a vital mechanism that keeps the mammalian circulatory system intact, is the generation of a fibrin clot through a series of stepwise reactions that are triggered by vascular injury. These reactions involve membrane surfaces of vascular endothelial cells and platelets, calcium, and plasma proteins (Table I.1).

The plasma proteins that participate in the coagulation cascade are either enzymes or their cofactors. These proteins circulate in blood in their inactive forms. Once the coagulation mechanism is initiated, they are converted to their active forms through proteolytic cleavage. The coagulation cascade contains two different pathways, namely intrinsic and extrinsic, which merge in a common pathway (Figure I.1). Intrinsic pathway is so called since it involves only coagulation proteins present in the plasma. Following a vascular injury, von Willebrand factor forms a bridge between the activated platelet and endothelial cells. This reaction, that is called platelet adhesion, is followed by the aggregation of a population of platelets at that site by fibrinogen. Thus formed, platelet plug prepares a phospholipid surface for the coagulation cascade to start. This pathway is initiated by the activation of factor XII and leads to the activation of factor X in a series of reactions (Figure I.1) [1]. However, the intrinsic pathway does not take into account a role for tissue factor and factor VII. Tissue factor is an integral membrane glycoprotein. Following a damage to the blood vessel walls, factor VII binds very tightly to the extracellular domain of tissue factor and starts the extrinsic pathway for the activation of factor X. The sequential reactions after activation of factor X constitute the common pathway, as a result of which fibrinogen is converted to fibrin and a stable fibrin clot is formed to repair the injured site [2] (Figure I.1.). Recent studies consider the extrinsic pathway as the

main pathway in coagulation and intrinsic pathway as an alternate one functioning in the maintenance of the fibrin clot [3]. The reactions involved in coagulation mechanism are regulated by a number of protease inhibitors such as tissue factor pathway inhibitor (TFPI), antithrombin III, protein C, and protein S.

Defects in any of the members of coagulation may lead to serious bleeding problems, leading to death in severe cases (Table I.1.).

TABLE I.1. Proteins involved in blood coagulation.

Common Name	Numeric Symbol	Related Disease
Fibrinogen	Factor I	Afibrinogenemia, Dysfibrinogenemia
Prothrombin	Factor II	Dysprothrombonemia
Tissue factor	Factor III	
Proaccelerin	Factor V	Parahemophilia
Proconvertin	Factor VII	Hypoproconvertinemia
Antihemophilic factor	Factor VIII	Hemophilia A
Christmas factor	Factor IX	Hemophilia B
Stuart factor	Factor X	Stuart (FX) deficiency
Plasma thromboplastin antecedent (PTA)	Factor XI	PTA deficiency
Hageman factor	Factor XII	Hageman trait (no hemorrhagic tendency)
Fibrin-stabilizing factor (FSF)	Factor XIII	FSF (FXIII) deficiency
Prekallikrein (Fletcher factor)	-	Fletcher factor deficiency (no hemorrhagic tendency)
High molecular weight kininogen	-	Fitzgeral factor deficiency (no hemorrhagic tendency)
Protein C	-	Protein C deficiency (thrombosis)
Protein S	-	
Antithrombin III (ATIII)	-	ATIII deficiency (thrombotic tendency)
Heparin cofactor II	-	
Tissue factor pathway inhibitor	-	
von Willebrand factor	-	von Willebrand factor deficiency

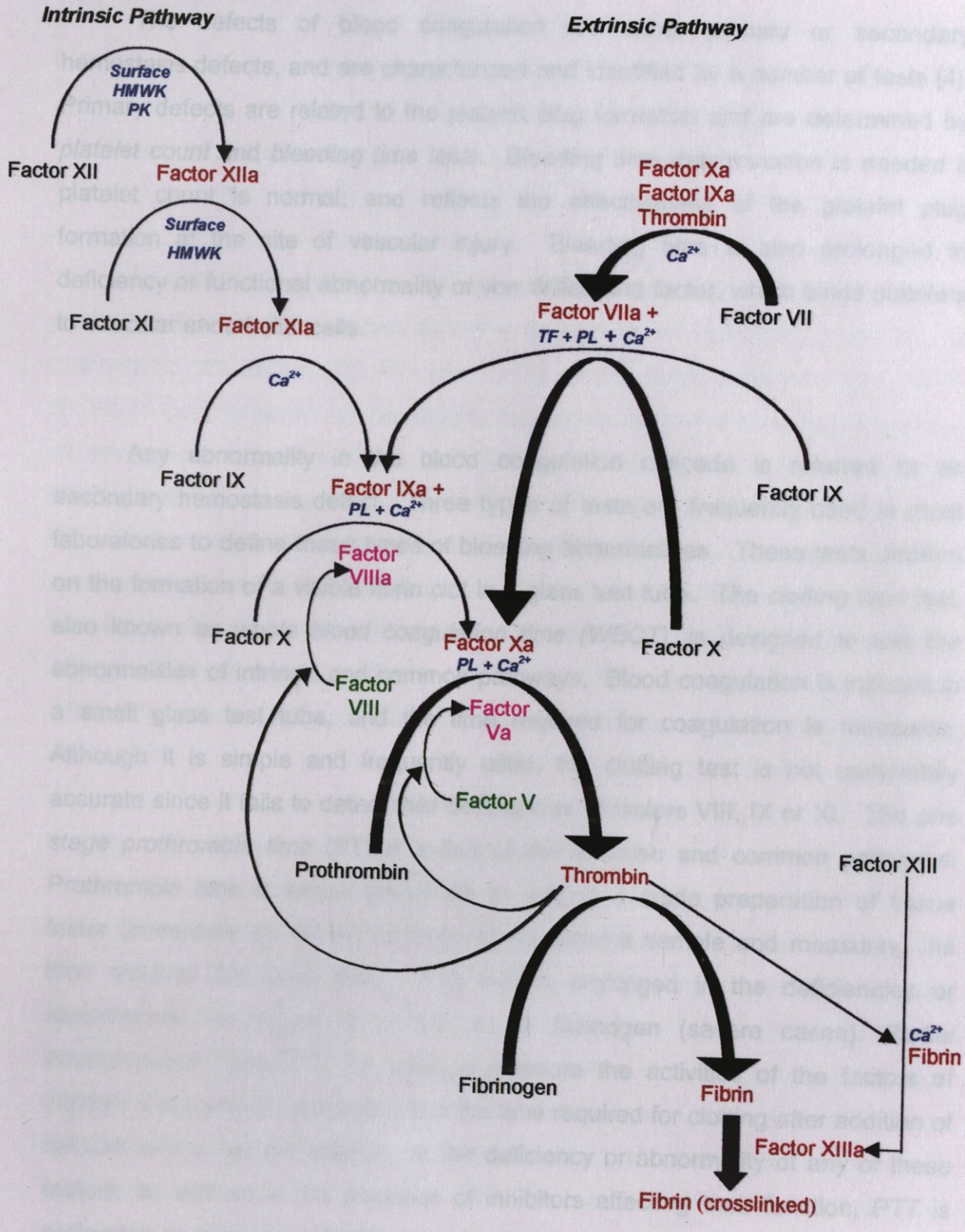


FIGURE I.1. Blood coagulation mechanism. The zymogens (black), their activated forms (red), the cofactors (green) and activated forms of cofactors (pink) are indicated in different colors. PK: Prekallikrein; TF: Tissue factor; HMWK: High molecular weight kininogen; PL: Phospholipid.

The defects of blood coagulation are either primary or secondary hemostasis defects, and are characterized and identified by a number of tests [4]. Primary defects are related to the platelet plug formation and are determined by *platelet count* and *bleeding time* tests. Bleeding time determination is needed if platelet count is normal, and reflects the effectiveness of the platelet plug formation at the site of vascular injury. Bleeding time is also prolonged in deficiency or functional abnormality of von Willebrand factor, which binds platelets to vascular endothelial cells.

Any abnormality in the blood coagulation cascade is referred to as secondary hemostasis defect. Three types of tests are frequently used in most laboratories to define these types of bleeding abnormalities. These tests depend on the formation of a visible fibrin clot in a glass test tube. The *clotting time* test, also known *as whole blood coagulation time (WBCT)*, is designed to test the abnormalities of intrinsic and common pathways. Blood coagulation is initiated in a small glass test tube, and the time required for coagulation is measured. Although it is simple and frequently used, the clotting test is not completely accurate since it fails to detect mild deficiencies of factors VIII, IX or XI. The *one stage prothrombin time* (PT) is a test of the extrinsic and common pathways. Prothrombin time is simply performed by adding a crude preparation of tissue factor (commonly an extract of brain) to the plasma sample and measuring the time required for coagulation. The test is prolonged in the deficiencies or abnormalities of factors II, V, VII, X, or fibrinogen (severe cases). *Partial thromboplastin time* (PTT) is used to measure the activities of the factors of intrinsic and common pathways. It is the time required for clotting after addition of calcium ions to the full plasma. In the deficiency or abnormality of any of these factors, as well as in the presence of inhibitors affecting their function, PTT is prolonged to more than 35-45 seconds, that is the normal range of this test. If PTT is performed in the presence of surface activating agents such as kaolin, silica, and phospholipid, it is called *activated partial thromboplastin time* (aPTT). These reagents are used in order to ensure that platelets are not rate-limiting and factors XI & XII are fully activated.

B. Hemophilia B

1. History of the Disease

Hemophilia was described as early as the second century CE. This disease had been thought to be caused by the deficiency of an antihemophilic factor until the midst of the century [5]. However, an interesting case was identified in 1947, in which, the plasma of one hemophilia patient had corrected the coagulation time of another patient's plasma [6]. In 1952, two independent studies successfully distinguished the two clinically identical hemophilias (hemophilia A and hemophilia B) and demonstrated that hemophilia B is caused by the deficiency of a coagulation factor other than factor VIII [7, 8]. They identified this coagulation factor and referred to it as plasma thromboplastin component (PTC). Immediately after this demonstration, a family with PTC deficiency was described and the disease was given the name "Christmas disease," the surname of the family [9].

2. Clinical Features and Diagnosis of the Disease

Today, hemophilia B is known as the third common hereditary bleeding disorder, following hemophilia A and von Willebrand factor deficiency. The disease is inherited in an X-linked recessive manner, is caused by the production of either reduced amounts or functionally defective forms of the coagulation factor IX (FIX) and affects one male in every 30000. One third of the cases are sporadic. It has been rarely described in females because of gross chromosomal abnormalities [10], non-random X inactivation or uniparental disomy [11, 12].

Hemophilia B is observed in three forms of clinical severity, namely mild, moderate and severe. The patients having the disease may show clinical features

as spontaneous bleeding, especially into joints and muscles, prolonged bleeding from minor cuts, and severe bleeding from lacerations. Repeated hemarthroses are formed by spontaneous bleeding into joints (including knees, elbows, ankles, shoulders, wrists and hips) usually leading to crippling joint deformities. Hematomas, which are hemorrages into subcutaneous connective tissues or into muscles (including calf, tight, buttocks, and forearm) are the other characteristics of the disease. Spontaneous bleeding, hemarthroses and hematomas are usually limited to patients with moderate and severe disease, while mildly affected patients may show symptoms only after clear traumas [13]. The clinical severity of the disease is mainly classified according to the bleedings into the joints per year; patients with severe hemophilia B have six or more spontaneous bleedings into joints in one year while mildly affected patients have infrequent bleedings only caused by dental extraction, and patients with moderate hemophilia B show intermediate symptoms [14]. The severity of the disease is also correlated with the antigen levels (FIX:Ag) and coagulant activity of factor IX (FIX:C) in the blood. Patients with coagulant levels of <1, 1-5, and 5-40 per cent of normal are usually severely, mildly, and moderately affected, respectively. The concentration of the FIX:C is <0.01 U/ml, 0.01-0.05 U/ml and 0.05-0.40 U/ml, for severely, mildly, and moderately affected patients, where U is the amount of FIX:C in one milliliter of pooled normal human plasma.

Hemophilia B, like other bleeding disorders, is diagnosed clinically by the tests mentioned in section I.A. *Bleeding time* is normal for hemophilia B patients, whereas *PTT* and *aPTT* are prolonged. The *clotting time* ranges from normal to long depending on the severity of the disease. *Prothrombin time* is normal except for the hemophilia B_m cases, a very heterogeneous subtype of the disease, which is characterized by the prolongation of the test only when performed with bovine brain extracts. Different types of hemophilia B_m patients have structurally abnormal and inactive forms of factor IX (FIX) that does not react with either of FXI, FVII-TF, or antithrombinIII/heparin in different forms of the disease.

3. Treatment of the Disease

The main goal in the management of a hemophiliac person is to improve his life span and quality. This includes mainly increasing the FIX amount to hemostatic levels and maintaining it. Today, the treatment of hemophilia B patients is mainly performed by replacement therapy, in which FIX is given to the patient through intravenous infusions. The amount of the FIX given to the patient is directly related to the severity of the disease as well as to the body weight of the patient and the biological half-life of FIX (18-24 hrs). To achieve hemostasis in a hemophilia B patient, his plasma FIX levels should be raised to 20-40 per cent at minimum.

Fresh-frozen plasma (FFP) is the first replacement product used to treat bleeding [15]. It is obtained from a single donor and contains one unit of coagulation factor in each milliliter of plasma. When used in 10-15 ml/kg of body weight, it is possible to raise the FIX levels at most to 10-15 per cent. This type of treatment is limited by the volumes administered since there is a risk of overloading the circulation in the patient, although there are cases in which higher levels are achieved with exchange plasmaferesis [16]. Thus, plasma therapy is practical for mild hemophiliacs but not for the severe ones since it is impossible to reach hemostatic or therapeutic levels in these patients. The advantage of plasma therapy resides in being more controlled in terms of blood-born diseases, since it is obtained from a single donor.

With the advent of blood fractionation technology in late 1960s, the treatment of the disease shifted to the use of new products concentrated in FIX. The first produced FIX concentrates, the prothrombin complex concentrates (PCC) [17], are prepared by the absorption of FIX as well as other vitamin K-dependent coagulation factors (from a plasma pool of thousands of donors) on DEAE-cellulose or DEAE-sephadex, followed by elution with phosphate or citrate buffer

and lyophilization. These concentrates contain an equal amount of prothrombin, FIX and FX but the amount of FVII varies in different preparations. With their tremendously increased FIX contents, these concentrates improved the life span and quality of the patients dramatically during late 1970s and early 1980s, until the appearance of blood-born diseases such as AIDS, hepatitis B and hepatitis C. The evidence of these life-threatening infectious diseases forced the development of safer blood screening techniques and sterilization procedures for the blood-derived products. The commonly used methods aim to destroy the lipid envelope of the infectious viruses and include dry heat (72 hrs at 80°C) [18], pasteurization (10 hrs at 60°C in a liquid medium) [19], and solvent/detergent [20]. Other methods are ultrafiltration and chromatography. The use of the above procedures alone or in combination improved the quality of blood-derived products so that after late 1980s the number of infected cases has decreased. In 1990s, however, prion protein diseases such as Creutzfeld-Jacob disease were added to the risk factors, although no cases (if not a few) has been reported yet among hemophiliacs. Another life-threatening side effect of crude FIX concentrates is the risk of thrombosis. Some of the coagulation factors in PCC might be activated during concentration procedures [21], or large quantities of the products might be used. Both cases trigger excess coagulation since PCC does not only contain FIX but other vitamin K-dependent coagulation factors. The development of highly purified FIX concentrates using monoclonal antibodies eliminates the risk of thrombosis but costs more than the crude preparations [22]. Inhibitor development against replacement therapy is another problem although rarely seen in hemophilia B patients (3 per cent). Such patients are treated both to stop bleeding and to induce immune tolerance. Some of the procedures followed are the use of recombinant FVIIa [23], repeated infusions of PCC [24], and suppression of antiFIX-antibodies by immunosuppressive agents.

Replacement therapy, so far explained, is used either on-demand or in prophylactic regimes. On-demand treatment, as the name implies, is the use of replacement therapy only when a bleeding problem occurs, or prior to any kind of surgery. The joint deformities of severe patients could not be prevented or

corrected using this type of treatment regime. However, another regime firstly started in Sweden in 1950s, aims to convert a severe patient to a moderately affected one. This regime is called prophylaxis, which is suitable for severely affected patients who did not develop any joint problems yet. The treatment starts at very early ages before the first bleeding episode is seen and includes infusion of the patient with low levels of FIX twice a week. Over 25 years, prophylaxis has been proved to be superior to on-demand treatment in maintaining normal joints, reducing joint bleeds and slowing the progress of already involved joint damages.

The cost of the replacement therapy is high. The price of the treatment is directly proportional to the product quality. The average amount of FIX used by hemophilia B patients in US is about 2000 U/kg/year. This number increases to 3000 U/kg/year in prophylaxis [25]. The cost of the prophylactic treatment could be decreased with the use of averagely purified concentrate products instead of highly purified ones. This is the case of İzmir protocol of prophylaxis [26] in which the cost is about 30000 USD/year for a severely affected patient, where on-demand treatment costs about 26000 USD. Thus, it is a matter of choice between financial and health problems.

Inspite of the side effects and risk factors involved, the development of replacement therapy increased the life expectancy of hemophilia patients from ages of 11 to sixties.

C. The Coagulation Factor IX

The coagulation factor IX is a vitamin K-dependent serine protease which shows a high degree of homology to other vitamin K-dependent serine proteases; factor VII, factor X, and protein C [27] (Figure I.2.). This homology shows that these proteins evolved from a common ancestor through gene duplication, gene modification, and exon shuffling. The comparison of the amino acid sequences of human FIX with other vitamin K-dependent coagulation factors, and FIX from eight mammalian species (sheep, pig, rabbit, rat, mouse, cow, dog, and Guinea pig), and the information that is provided by various mutations observed to date, helped to explain the genotype/phenotype relationships and functionally important regions of FIX. An amino acid in the FIX sequence is considered as *generic* if it is conserved in FIX and other closely related coagulation serine proteases, as *partially generic* if it is conserved in FIX and in one or two of closely related coagulation serine proteases, and as *FIX specific* if it is conserved in FIX of other mammalian species [28] (Figure I.3.).

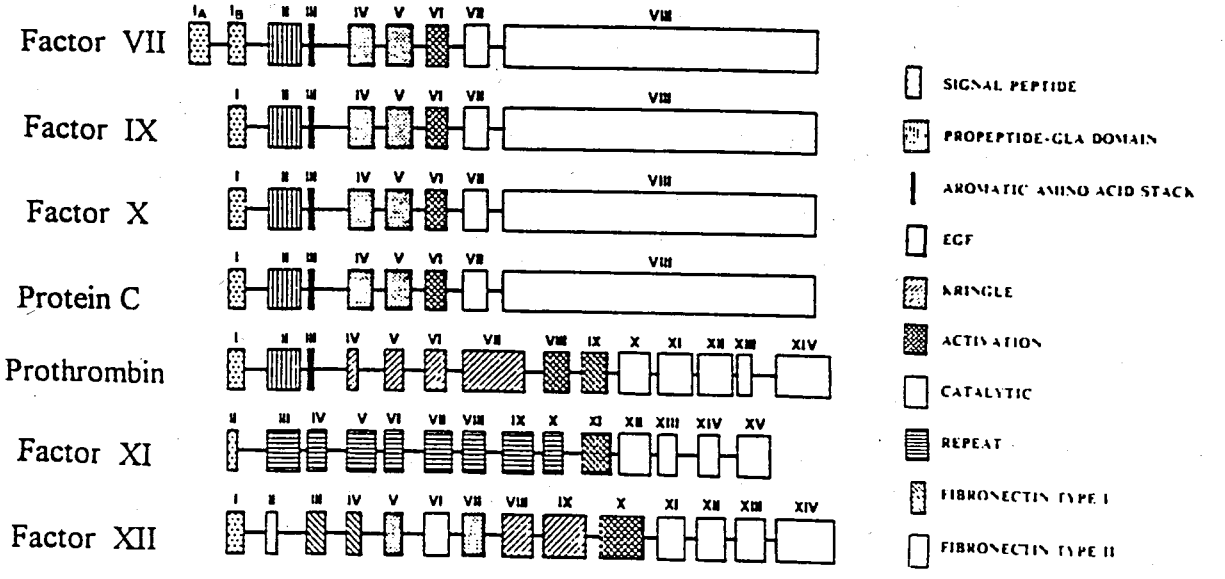


FIGURE I.2. The homology of the blood coagulation serine proteases.

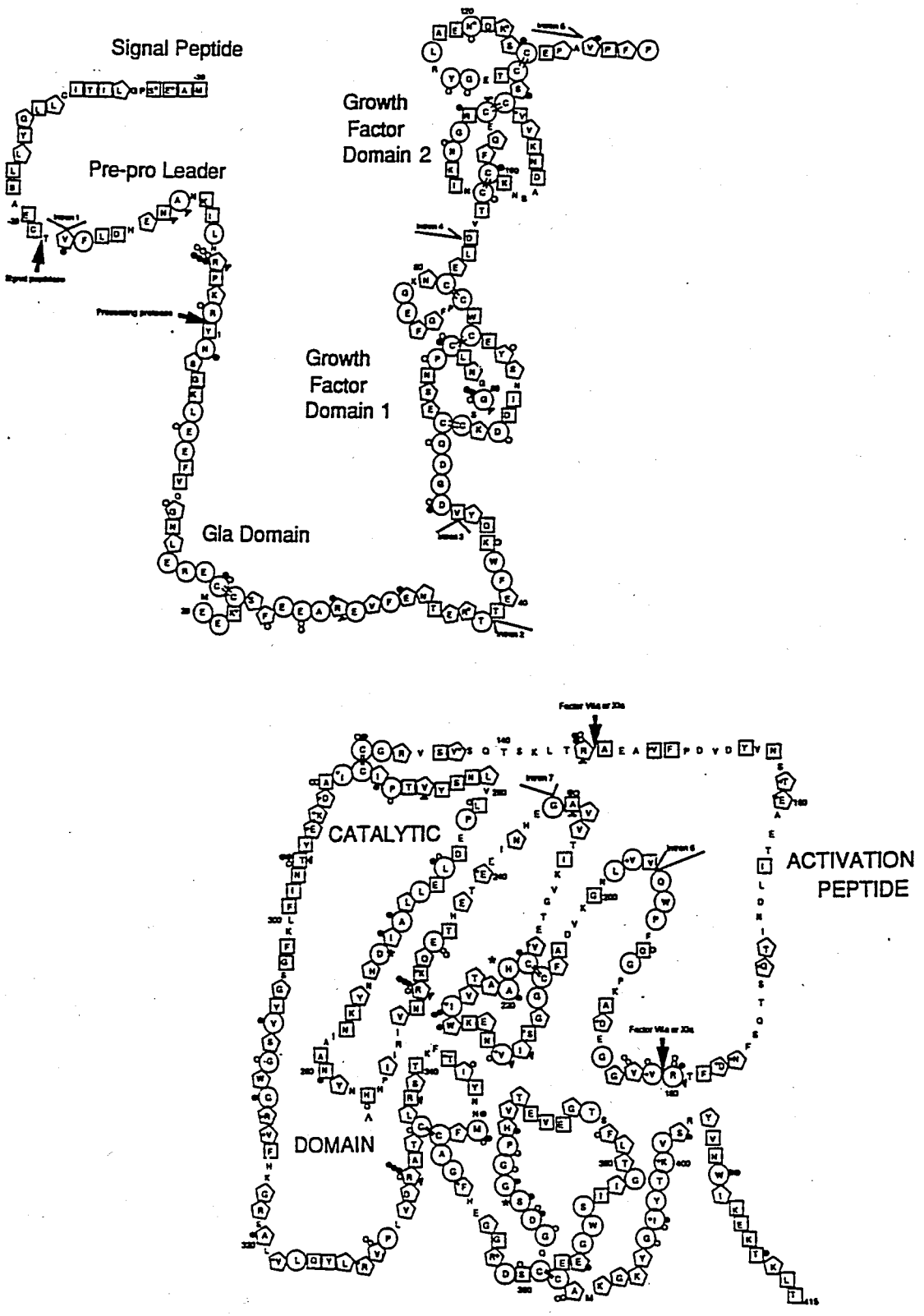


FIGURE I.3. Degree of conservation of FIX residues. Circles represent *generic*, squares represent *factor IX-specific*, and pentagons represent *partially generic* residues.

Factor IX is responsible for the activation of factor X in the presence of factor VIII (the cofactor of FIX) and Ca^{2+} on the membrane surfaces (Figure I.1.). It is specifically synthesized in liver as a 56.8 KDa and 461 amino acid long preprofactor IX. The FIX is a modular protein, and almost every domain is coded by a separate exon of the gene (Figure I.4.).

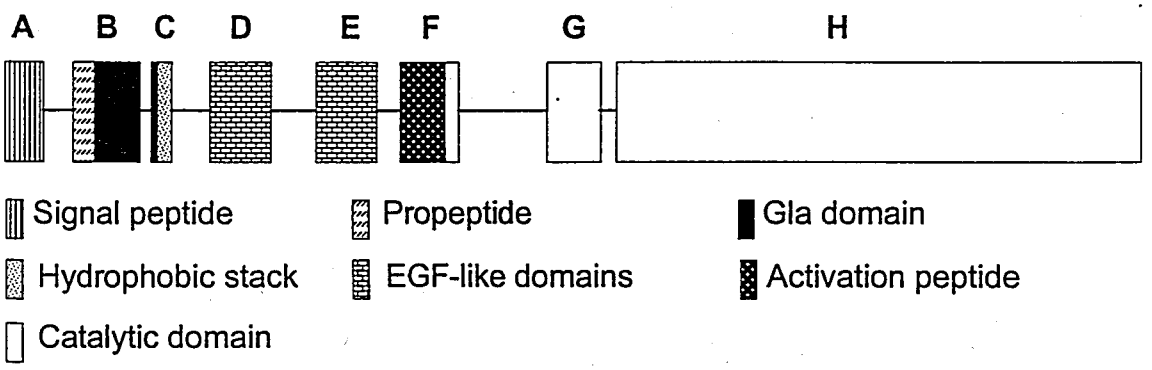


FIGURE I.4. The schematic representation of the FIX gene and its corresponding modular protein structure [29]. The letters A-H represent the exons of the gene.

It is mainly composed of three parts; a 29 amino acid long signal peptide (residues -46 to -18) which is coded by exon A, a 17 amino acid long propeptide (residues -17 to +1) which is coded by 5' portion of exon B, and a 415 amino acid long zymogen.

The *pre sequence* (signal peptide) leads the protein to rough endoplasmic reticulum (ER) lumen and is then cleaved by a signal peptidase. Then, the profactor IX undergoes some post-translational modifications, namely O-glycosylation of residues Ser61 and Ser53, respectively, as well as the N-glycosylations of Asp157 and Asp167, partial β-hydroxylation of the Asp64 residue and γ-carboxylation of the 12 Glu residues [30]. Carboxylation and hydroxylation

of the protein is completed in ER, but glycosylation process is finished in Golgi complex.

The α-helical secondary structure of the *propeptide* [31] creates a recognition surface for the vitamin K-dependent microsomal carboxylase which γ-carboxylates the glutamic acid (Glu) residues of the following *Gla domain* and *hydrophobic stack* [32]. After the profactor IX is post-translationally modified, the propeptide is cleaved by a specific peptidase and the zymogen is secreted into the blood stream.

The 415 amino acid zymogen is composed of five domains. *Gla domain*, constitutes the amino terminal of the protein. This domain owes its name to the 11 γ-carboxylated glutamate (Gla) residues it contains. Two different mechanisms have been proposed for the membrane binding of FIX through this domain. The first one says that highly negatively charged dicarboxylic acid side chains of these Gla residues bind Ca^{2+} ions, which are suggested to form an electrostatic bridge between factor IX and a phospholipid surface [33]. However, it has been proven that binding of Ca^{2+} ions to the Gla residues induce sequential conformational changes in this domain and defines its tertiary structure for its binding to the membrane surfaces [34]. Now, there is evidence that Mg^{2+} ions are also involved in the formation of the tertiary structure of this domain, thus in the functional conformation of the protein [35] and the binding of these ions are depicted to some yet unidentified residues in Gla domain different from the ones interacting with Ca^{2+} ions naturally [36]. It was suggested in a study that, the interaction of the first residue of Gla domain with residues 21 and 22 following the conformational changes probably defines the critical phospholipid binding surface of the protein, and a hydrophobic patch, including residues 1-11, represents a component of phospholipid binding site through insertion into the phospholipid bilayer [37]. Different from these observations, the residues 3-11 of Gla domain were defined as responsible for binding to endothelial cells probably through a receptor interaction [38]. Two recent studies found that the binding site of these residues is

collagen IV; an extracellular matrix protein that is one of the major components of the basement membrane of endothelial cells [39, 40]. These observations agree well with the involvement of FIX in both intrinsic and extrinsic pathways of blood coagulation. In a study, a FIX variant having substitution of a Gly residue, in Gla domain, for Arg was shown not to have normal activity even in the presence of its cofactor FVIII; this suggests that the structural integrity of the Gla domain is important for the binding of FVIIIa by FIXa [41].

A highly conserved eight amino acid long *hydrophobic domain* contains the last Gla residue. This domain also has a conserved Phe-Trp-X-X-Tyr motif that was shown to be required for the Ca^{2+} dependent conformational changes in the protein [42]. This observation is in accordance with that of Jacobs *et al.* [43], who suggested that this domain together with the Gla domain (residues 1-47) form a functional membrane binding site. Another study showed the importance of the Phe residue of the conserved motif in stabilizing the phospholipid binding conformations as well as the influence of this residue on the function of following EGF-like domain [44].

The *first EGF-like domain (EGF1)*, so called because of its similarity to human epidermal growth factor, contains the post-translationally modified β-hydroxyaspartate residue 64 which forms a high affinity Ca^{2+} ion binding site together with residues 47 and 50 within this domain [45]. A study with various mutants of the EGF1 domain revealed the importance of this domain in the activation of FIX through extrinsic pathway but not through intrinsic pathway [46]. Moreover, scientists performing the same study suggested that, this domain is also important in FX activation by "tenase" complex formation on phospholipid surfaces according to the observations of low coagulant activities of the activated EGF1 mutants. There are observations from independent studies that support this suggestion. The residue 78 in the EGF1 domain was suggested to maintain the integrity of FVIIIa light chain binding site by linking both EGF domains through forming a salt bridge with residue 94 in EGF2 [47]. In another study, scientists

proposed a mechanism (depending on the X-ray structure of porcine FIXa and information about the mutant FIX proteins) for the formation of tenase complex through the interaction of the EGF1 domain with Gla domain via a salt bridge that is formed following metal dependent conformation changes[48]. Two glycosylation sites are present in the 1st EGF-like domain.

The *second EGF-like domain (EGF2),* follows the first one, and no other function than the one mentioned above has been reported yet. The six disulfide bonds formed in the two EGF-like domains suggest the importance of these domains in the stability of the protein.

The following domain is the *activation peptide*. The cleavage of the dipeptide bonds between residues Ala145-Ala146 and Arg180-Arg181 upon interaction with FXIa in the presence of Ca^{2+} ions or with FVIIa in the presence of tissue factor, phospholipid and Ca^{2+} ions gives rise to the excision of this domain [49]. The excision of the activation peptide is a two step process, in which a non-functional intermediate FIXaα structure is firstly formed upon cleavage of the 145-146 bond. Following the formation of FIXaα, the light chain of the protein is linked to the heavy chain that is composed of the *catalytic domain*, via a disulfide bridge between Cys132 and Cys289. The completely functional FIXaβ is formed following the cleavage of the 180-181 bond. The activation peptide also contains the two N-linked carbohydrates (Figure I.5.).

The *catalytic domain* includes three substrate binding pocket residues (Asp359, Gly386, and Gly396), and three active site residues (His221, Asp269, and Ser365), 'the catalytic triad', that is required for the activation of factor X in the presence of Ca^{2+} ions, phospholipid and factor VIIIa. The most important residue for substrate binding is Asp359, which binds non-covalently to the Arg side chain at the activation cleavage site in FX. The free amino-group of the heavy chain (Val181) forms an ion pair with Asp364 (the residue next to the active Ser365) and

therefore allows the activation of the hydroxyl group of the Ser365. Studies on trypsin and thrombin have shown the importance of the formation of this ion pair, and the evidence for FIX case came in 1988 [50]. Activation of the FX is achieved with the help of the charged side chains of other active site residues, His221 and Asp269. Recently, two sites have been defined in this domain, between residues 301-303 and 333-339, which interact with the heavy chain of FVIIIa, thus playing a role in the FVIIIa-dependent stimulation of FX activation [51].

FIXa is inhibited by antithrombin through its covalent interaction with Ser365 at the active site [49]. The rate of the formation of this complex is enhanced by the presence of heparin.

D. The Factor IX Gene

1. Structure

The gene coding for the human coagulation factor IX was first cloned in 1982 through utilizing the amino acid sequence of bovine factor IX [52], and was then localized to the distal part of the X chromosome, Xq27, in 1984 [53]. The complete sequence of the gene was determined in 1985 [54].

The factor IX gene spans at least a 34 kb region [54]. The exact size has not been determined yet because of the probable presence of regulatory elements within the sequences flanking the gene. The factor IX gene is composed of eight exons, the lengths of which vary from 25 bp to about 2000 bp (Figure I.4.). The human factor IX mRNA is about three kilobases in length and is composed of 205 bases for 5' UTR, 1386 bases for the preprofactor IX, and 1392 bases for 3' UTR.

The 3' sequence of the gene contains a polyA tail addition site, 1.4 kb 3' to the stop codon.

The factor IX gene has several repetitive sequences. Four of the five Alu repeats are found in the gene within the introns, and one in the 3' flanking sequence. Moreover, one Line-1 element is found in the 5' flanking region and one in intron 4 [54]. There are also two defined cryptic RY(i) repeats (purine-pyrimidine tandemly repeated dinucleotides) in intron 1 and in the 3'UTR of the gene [55].

2. Regulation of Expression

The FIX gene is expressed specifically in hepatocytes although illegitimate expression in other tissues has been reported [56]. The expression of the gene is also age dependent as has been shown in mice [57], as well as in limited human [58] experiments. The FIX level is 3-5 per cent of adult level until third trimester of the gestation, 43 per cent at birth, 75 per cent during childhood. These levels increase to 100 per cent into adulthood and continue to rise during lifetime by one per cent per year [59]. This age-dependent increase in FIX levels is directly related to the FIX mRNA levels, therefore directly related to the up-regulation of the FIX gene promoter.

There is no definite promoter region for the FIX gene. The putative promoter region does not have a typical TATA box, but a six bp TCAAAT sequence, located between nucleotides -187/-182. This sequence was assigned as the TATA box of the gene [60]. The CCAAT box-like sequence, AGCCACT, lies on nucleotides –238/-232 [54]. The major transcription initiation site for the gene is suggested to be at or around the nucleotide numbered -176 [60], which is much more upstream to the first transcription initiation site defined at nucleotide numbered +1 [61].

There are also other defined transcription initiation sites [62], a situation that is concordant with the lack of a typical TATA box.

The putative promoter region is under the control of some *cis* acting enhancer (Table I.2.) and silencer elements. Two functional consensus silencer elements, at positions -1680 (ATCCTCTCC) and -1621 (CAATGGTT), and some silencer-like elements at nucleotides –726, -793 and –1467 were identified. Additionally, a strong promoter activity in reverse direction was observed between nucleotides –700 to –750 [63]. The exact function of this promoter is not certain yet although it has been reported to negatively affect the expression of the gene. Intron 1 of the gene has also been reported to have slight regulatory effects on the gene expression [64].

TABLE I.2. The transcription factors, controlling the expression of the FIX gene.

Name	Location	Reference
CCAAT/Enhancer Binding Protein (C/EBP) & D-site binding protein (DBP)	-219/-202	[65]
Liver Nuclear Factor 1 (NF-1L)	-99/-77	[66]
Androgen Response Element (ARE)	-34/-23	[67]
Hepatocyte Nuclear Factor 4 (HNF-4)	-27/-19	[68]
Unidentified	-15/-1	[65]
CCAAT/Enhancer Binding Protein (C/EBP)	+4/+19	[66]

There is a specific type of hemophilia B, hemophilia B Leyden. These patients have low levels of FIX in childhood but their FIX levels increase following puberty. The sequence lying between nucleotides –34/+19 in the gene is known as the Leyden specific (LS) region, and the mutations defined in this region (at nucleotides –26, -21, -20, -6, -5, +6, +8, and +13) [69] give clues of the post-pubertal phenotypic recovery of hemophilia B Leyden patients.

Several groups have proposed various contradictory mechanisms about the positive regulation of the FIX gene expression. One such mechanism suggests a role for steroid hormone activity and says that the direct interaction of androgen receptor with ARE is responsible for post-pubertal phenotypic recovery [67]. Support for this mechanism comes from mutations observed at the HNF-4 binding region, and from hemophilia B Brandenburg phenotype, in which no phenotypic recovery is observed after puberty although the patients have a mutation in the LS region (at nucleotide –26), more specifically in a region overlapping ARE and HNF4 binding site. A recent study showed that AR binds to ARE site and is also in association with other proteins binding to this promoter [70]. Another group agrees with steroid hormone activity, but suggests that the interaction of AR with ARE is not direct, but happens through an unidentified protein [71].

Another mechanism, however, stresses the importance of C/EBP mainly in FIX gene regulation, as well as its synergistic interaction with DBP in phenotypic recovery of hemophilia B Leyden patients. C/EBP is known to be expressed during fetal development and stays constant in concentration, while DBP starts to be expressed in very low levels pre-pubertally and reaches maximum expression levels only in adulthood. According to this information, this mechanism suggests that the developmental expression of DBP along with its synergistic interaction with C/EBP (at -219/-202) suggests a potential role for DBP in contributing the post-pubertal recovery as well as the lifetime increase of FIX [65, 72]. In a continuation of this study, another transcription factor GA-binding protein (GABP) was suggested to be involved in the interaction of C/EBP and DBP at site -219/-202 [73]. Further support to the hypothesis suggesting the importance of C/EBP in FIX gene regulation comes from the non-expression of FIX in C/EBP knockout mice [74].

Neither of the mechanisms yet, can fully explain the regulation of the FIX gene and the phenotypic recovery observed in hemophilia B Leyden patients.

E. Normal Variants of the FIX Gene

Several polymorphic sites have been detected either within the FIX gene or in the flanking sequences of the gene. The RFLP sites which are found within the gene are *Xmn*I [75] (intron 3), *Taq*I [76] (intron 4), *Msp*I [77] (intron 4), and *Mnl*I [78] (exon F). The ones that are present in the flanking sequences are *Mse*I [79] and *BamH*I [80] (5'UTR) and *Hha*I [81] (3'UTR). To date, *Mnl*I is the only exonic polymorphism identified and it has two alleles containing either an Ala or a Thr at residue 148, Thr allele being more common. The polymorphism originally is known as Malmö but since the nucleotide change creates a second *Mnl*I recognition site in the PCR product of the region, it is also called *Mnl*I. *BamH*I, *Mse*I, *Hha*I, *Xmn*I and *Taq*I RFLPs are also characterized easily, because of the created recognition sites for appropriate restriction endonucleases, by restriction enzyme analysis. Recently, a new -793A/G dimorphism was identified in the promoter region of the FIX genes of Caucasian population [82], which neither creates nor abolishes a recognition site for any restriction endonuclease.

Besides these dimorphisms, there are two other polymorphic regions in the FIX gene. They both are alternating purine-pyrimidine repeats [RY(i)], which lack long tandem identical repeats, a reason to name them as cryptic RY(i) [cRY(i)]. One of them is located within the first intron. It was initially described as a *Hinf*I/*Dde*I polymorphism which is identified by a 50 bp deletion/insertion [83], but then found to be multiallelic rather than biallelic in a study with a sample size of about 1700 individuals [84]. The variety comes from two repeating units called A and B that are repeated several times to create novel alleles. Unit A is 24 bp long and B is 2 bp longer since it is A with an additional 3' (AT) (Figure I.5.). Seven different alleles of the form $A_{0-4}B$ and $A_{1-3}B_2$ have been identified in this study. The seven alleles of this hypervariable region are found to be very specific to races and ethnic groups analyzed (Caucasians, Hispanics, African-Americans and Asians), of which AB is the common allele in all ethnic groups. B allele is only found in Blacks, whereas $A_{1-3}B_2$ set of alleles are found only in Caucasians but not

in Asians and Blacks Recently, one more allele was identified in the form of A_5B in Brasilian Black population [85]. Another cRY(i) was identified in the 3'UTR of factor IX gene, which was also race specific. To date, four different alleles have been identified. Allele II is the most frequent one found in all populations. Allele I is absent in Asians whereas allele III is rare or absent in Caucasians or Blacks [86].

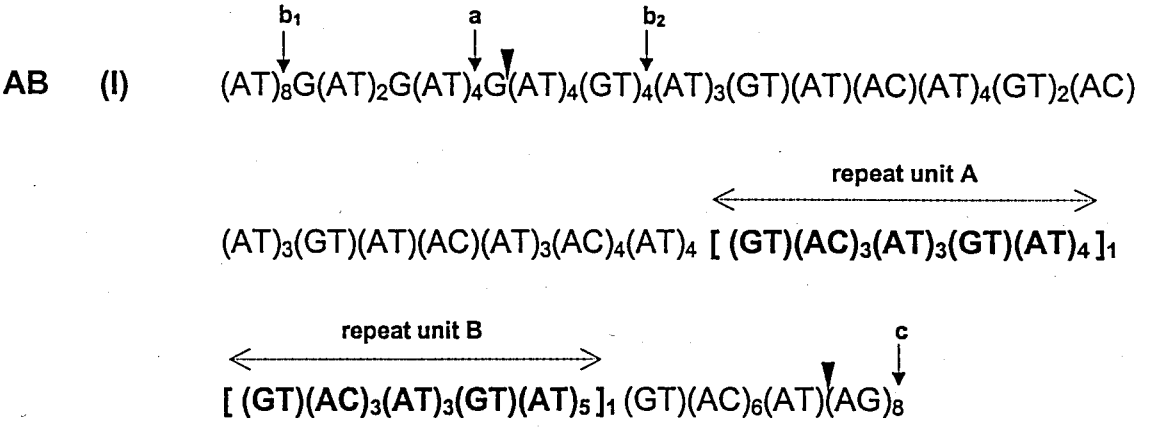


FIGURE I.5. Intron 1 cRY(i) and its repetitive flanking sequence (between arrowheads). The numbers after the brackets reflect the number of A and B segments in each polymorphic allele. Only AB common allele is given in this figure. **b1**, **a**, **b2** and **c** show other polymorphic dinucleotide repeats at that region.

All polymorphic sites are in linkage disequilibrium with each other except *Hha*I [87]. Similar to RY(i)s, the allelic and heterozygote frequencies of these polymorphisms show a significant variation among different ethnic groups. For example, African population is highly polymorphic for *BamH*I whereas populations of Asian origin are highly polymorphic only for *Hha*I [88]. The analysis of the Turkish population for these polymorphisms showed that our population is highly polymorphic for *Hha*I and *Hinf*I/*Dde*I and less polymorphic for *Mnl*I, *Taq*I and *Xmn*I,

a pattern that is very similar to the heterozygosity patterns of European populations [89, 90].

The total result of the analysis of these six polymorphic regions forms the haplotypes of the individuals. Haplotype analysis is used for linkage studies in DNA diagnosis and in the case of a recurrent mutation, to determine whether the mutation has either an independent origin or a common ancestor.

F. Abnormal Variants of Factor IX Gene

Direct sequencing for the identification of mutations for relatively small genes is possible as in the case of the factor IX gene. Although it is perfectly efficient in identifying the mutations, when a large sample size is considered it becomes tedious, time consuming and expensive. Thus, a need for the use of screening techniques arises in order to detect the mutations rapidly and easily.

Hemophilia B is very heterogeneous both in clinical severity and at the molecular level. Therefore, unlike the disorders that are caused by only one or a few number of mutations (sickle cell anemia and β-thalassemia), scanning methods for identification of known mutations are not useful in detecting factor IX mutations. Rather, several PCR based-rapid mutation screening methods that enable detection of unknown mutations are used. The most frequently used ones are the *a*mplification and *m*ismatch *d*etection (AMD) [91], *s*ingle strand *c*onformation *a*nalysis (SSCA) [92], *d*enaturing *g*radient *g*el electrophoresis (DGGE) [93], heteroduplex formation analysis [94], and *did*eoxy *f*ingerprinting (ddF) [95].

1. Types of Mutations Affecting the Gene

The invention of polymerase chain reaction [96] has lead to the identification of FIX mutations all over the world, so that a database (collecting all of the data except for the gross gene rearrangements) was established in 1990, and published as a paper in *Nucleic Acids Research* [97]. This database has been updated annually with the addition of new patient entries and continued to be published as a paper in the same journal until 1996, in which, an internet site [69] was established because of the enormous amount of data collected. The data of 1918 patient entries is available in the very recent version of the database. Of these, 689 cases are unique molecular events while the remaining 1229 are recurrent mutations. The frequency of the mutations affecting the gene (caused by single base substitutions, and small deletions/insertions), except for gross gene arrangements, are indicated in Table I.3.

TABLE I.3. The effect of mutations on the FIX gene.

Type of Mutation	Frequency of Mutation Type (%)
Missense	68
Nonsense	14
Splice-site	6
Cryptic splice	3
Promoter	3
Frameshift	5
In-frame deletion	1

Heterogeneous clinical presentation of hemophilia B is a result of extremely heterogeneous mutations affecting the FIX gene. These mutations form three hemophilia B phenotypes in terms of FIX:Ag and FIX:C levels. The patients with no FIX:Ag are designated as cross-reactive material negative (CRM^-). The

mutations giving rise to this kind of phenotype are the ones impairing the synthesis, secretion and stability of FIX. The other phenotype is cross-reactive material positive (CRM^+), and such patients have nearly normal FIX:Ag levels while having reduced FIX:C levels. These patients have mutations interfering with the function of FIX. The intermediate of these two is cross-reactive material reduced (CRM^{red}) patients, who have proportionally reduced levels of both FIX:Ag and FIX:C. Such patients usually have mutations affecting the synthesis, secretion, stability and function of FIX.

The majority of the mutations affecting the FIX gene are single nucleotide changes that are located throughout the gene from promoter to the end of the coding region. The mutations of the promoter region possibly disrupt the recognition sequences for several specific gene regulatory proteins and result in the reduced synthesis of coagulation factor IX. Until now, the point mutations of the putative promoter are only observed in the Leyden specific region. The majority of these point mutations are transitions and transversions, with the exception of two one-base deletions. All point mutations affecting the promoter region, except for the –26 mutation, result in a severe-moderate phenotype in childhood which recovers following puberty.

Some nucleotide changes occur at evolutionally conserved donor-splice (GT) and acceptor-splice (AG) consensus sequences and some of them create cryptic splice junctions, thus affect the processing of factor IX primary RNA transcript. Moreover, the mutations that affect the sequences flanking the splice sites also cause defective RNA processing by interfering with the assembly of spliceosomes. Several numbers of splice-site mutations have been detected in all of the exon-intron boundaries and mutations creating cryptic splice sites have been detected at seven different positions for several times.

Missense mutations causing amino acid substitutions in the coding region of the FIX gene pinpoint structural features that are important for the secretion, stability, and function of the protein. For example, mutations affecting cysteine residues usually result in CRM^{red} phenotypes, which confirms the importance of these residues in keeping the protein stable through the disulfide bridges they form. Interestingly, a mutation affecting the Cys18 residue results in a CRM^{+} phenotype, showing that this residue and the disulfide bridge it forms does not contribute to the stability of the protein [69]. Other types of mutations affecting the coding regions are nonsense and frameshift mutations. Nonsense changes and small deletions and insertions causing frameshifts result in defective translation of FIX and produce an unstable truncated protein. Small in frame deletions and insertions are rarely described.

Gross and complex gene rearrangements are rare in hemophilia B. Large deletions including complete and partial gene deletions have been described but no contiguous gene syndromes have been reported until recent dates. However, there is an interesting report in which both FIX genes and DBL proto-oncogenes are deleted in two patients [98]. Interestingly, the only observed symptoms are the ones of hemophilia B in these patients. The complete and partial deletions account for more than 50 per cent of inhibitor developing patients [99]. Insertions and gene duplications are also rarely reported [100, 101]. Deletions and insertions together account for about 15 per cent of independent mutations. In a study of sample size 290, the frequency of large deletions (>2 kb) was calculated as six per cent of the independent cases [102].

2. Hot-Spots of the Mutations

Single nucleotide changes are of two type: transitions and transversions. In the case of FIX gene, a great majority of the single nucleotide changes are transitions. Despite the heterogeneous nature of FIX mutations, there are several

sites where the mutations occur frequently. The CpG dinucleotides in the coding regions of the FIX gene have been shown to be real hot-spots of point mutations. There are 21 CpG sites in the FIX gene: 20 in coding regions and one in the promoter region. The distribution of these sites throughout the coding regions of gene is shown in Table I.4.

TABLE I.4. The distribution of CpG sites throughout the coding regions of gene. The slash between amino acid numbers indicates that C and G nucleotides are shared by two adjacent amino acids.

FIX Domain	Exon	# of CpG sites	Location of CpG sites
Signal peptide	A	2	-44; -44/-43
Propeptide Gla	B	3	-11/-10; -4; 29
Hydrophobic stack	C	-	-
EGF-1	D	1	59/60
EGF-2	E	2	95/96; 116
Activation peptide	F	2	145; 180
Catalytic	G	2	210/211; 232/233
	H	8	248; 252; 276/277; 284/285; 296; 333; 338; 403

The mechanism that causes transitions at CpG dinucleotides is spontaneous deamination of a methylated cytosine to give a T residue. Thus, CpG dinucleotide is converted to either a TpG or a CpA dinucleotide depending on the site of deamination; sense or antisense strands, respectively.

It is now becoming clear that the high number of recurrent mutations at some CpG dinucleotides, particularly those causing mild disease are the result of, at least in part, by founder effects. Population based studies on US and Canadian

Caucasian populations of primarily European descent showed that $Thr^{296}\rightarrow Met$, $Gly^{60}\rightarrow Ser$, and $Ile^{397}\rightarrow Thr$ mutations together account for about 25 per cent of all mutations observed [103]. Moreover, large pedigree and haplotype analysis among Amish population, in which hemophilia B is relatively frequent, strongly suggested that the 31008 C>T ($Thr^{296}\rightarrow Met$) was the mutation of this population dating to at least 1700s. Another hot-spot of mutations, which is thought to be partially result of a founder effect, is G-6A [104]. All of the four mutations are transitions at CpG dinucleotides except for $Ile^{397}\rightarrow Thr$, which is a 31311 T>C transition. Besides nucleotide 31311, there are 7 other non-CpG sites at which mutations occur at least more than five times. They are nucleotides 13, 17810, 30992, 31103, 31127, 31287, and 32528 [69]. No founder effects have been reported about the mutations on these sites yet.

G. The Hope for the Cure of Hemophilia B: Gene Therapy

The development of recombinant DNA technology, and the cloning and characterization of FIX gene pioneered the expression of FIX cDNA in various mammalian cell types for either as a recombinant protein for replacement therapy or for the ultimate cure of the disease, namely gene therapy. Factor IX presents some challenges to recombinant technology because of its complex and extensive post-translational modification processes. The initial experiments on obtaining human FIX *in vitro* presented only low level expression of FIX. The expression of recombinant FIX (rFIX) in Chinese hamster ovary cells secreted the protein into the medium upon co-transfection with a propeptidase [105]. Based on this study, functional and highly purified rFIX concentrates are produced, characterized, purified and preclinically studied on mice, rabbit and dog models recently. The structural, functional and safety properties of these products was found comparable to monoclonal plasma-derived FIX concentrates and now are commercially available [106].

Although being efficient in curtailment of the complications of the disease, replacement therapy does not cure the disease. The gene therapy studies for hemophilia B have been carried out either *ex vivo* or *in vivo*. A number of cell types such as primary or immortal fibroblats , capillary endothelial cells, myoblasts, keratinocytes have been used as target cells to test their ability to synthesize, post-translationally modify and secrete FIX [107]. Moreover, several types of vectors have been used, among which retroviral vectors have been of major interest for *ex vivo* studies. These vectors, constructed by several groups, differ in their promoters, the mostly used ones have been 5' LTR sequence itself and cytomegalovirus promoter. Until recent dates, the results of *in vitro* experiments were promising, but upon injection or transplantation into animal models, plasma levels was low and short term. The main reasons for this could have been down-regulation of the vector promoter after transplantation of FIX producing cells *in vivo*, cell death because of improper transplantation, or problems in transport of protein to plasma. The use of keratinocyte-specific elements in the transcriptional control of rFIX in mice keratinocytes resulted in detectable levels in plasma [108]. Another study revealed similar results through an up regulation of the cytomegalovirus promoter/enhancer with the use of different keratinocyte specific regulatory elements [109]. A recent study concentrated on the short-term nature of the transgene expressions *in vivo*. Transduced keratinocyte stem cells, producing high amounts of FIX in culture, were grafted onto mice under a silicone transplantation chamber and obtained detectable plasma levels. The further improvement of experiments with the use of MFG retroviral vector also further improved results and the detectable plasma FIX levels were maintained over one year [110]. There are commercially available immunoprotective cell transplantation devices recently [111].

The recent encouraging *ex vivo* studies and the presence of either natural canine animal models [112] or knock-out mice strains [113] lead scientists to continue *in vivo* studies more commonly. The first prospective study of *in vivo* gene transfer was performed by direct injection of retroviral vectors carrying canine FIX cDNA into portal vein of hemophilia B dogs. The dogs were partially

hepatectomized prior to injection of the vectors to induce transduction [114]. Although the produced FIX levels were 0.1 per cent of normal canine FIX plasma levels, whole blood clotting time and PTT values decreased significantly, and stayed constant over 24 months. Although promising, the hepatectomic surgery is one of the limiting factors of this study when therapy for humans was considered. Thus, the use of other retroviral vector systems that can transduce non-dividing cells, such as lentiviral vectors, or the use of higher titer retroviral vectors would be promising in hemophilia gene therapy. Another obstacle is the low level of expression, which is far away being therapeutically efficient. However, the use of the stronger promoters and enhancers specific to the used cell type can also improve the results obtained.

In 1994, the above experiment was repeated with adenoviral vectors [115]. These are high titer vectors, do not need dividing cells for transduction, highly tropic to hepatocytes through portal vein injection. The experiment yielded 2-3 times of wild-type FIX level in plasma, which normalized WBCT and PTT of the hemophiliac dogs, but the limiting factor was that the FIX levels returned back to the pre-treatment levels in 2 months. This is a case also seen in mice experiments with adenoviral vectors infused by intramuscular injection targeting the myoblasts [116]. The main reason for this transient expression with these vectors is cell-mediated and humoral immune responses against viral antigens. Another reason is the episomal nature, therefore decreased stability, of viral DNA. Therapy with this vector system could be improved by using immunosuppressive agents or by further deletion of the viral genes producing the immune responses [117].

Recently, use of another type of vector, adeno-associated virus (AAV), is a great promise for gene therapy because of its ability to transduce non-dividing cells without eliciting immune responses. The only disadvantage of this vector is its dependence to a helper adeno virus for replication. Recombinant AAVes (rAAV) contain the engineered expression casette for the transgene, flanked only

by inverted terminal repeats but not any other viral genes. A recent study achieved the stable expression of therapeutic levels of human FIX in an immuno-deficient mouse model for more than one year through intramuscular injection of the recombinant vector. However, no detectable plasma hFIX levels were observed after the repetition of the same experiment in an immuno-competent mouse strain because of antibody formation against the human protein [118]. A similar result was obtained in another study performed on hemophilia B dogs [119]. Moreover, the contamination of a lymphatic node with the helper adenovirus led them to start studies on optimizing the production of rAAVes not dependent on infectious helper viruses. The results of studies suggested the longer expression of human FIX could be achieved by avoiding cross-species experiments. Proof came from a recent hepatic gene transfer study. Hemophilia B mice and dog models were given AAV vectors carrying human and canine FIX genes, respectively. The expressed hFIX corrected the bleeding of mice over a period of 17 months, despite the low presence of antibodies against hFIX. The same amount of vector given to dog models partially corrected the WBC time and reduced the spontaneous bleedings about 8 months and no immune response was observed [120]. The more successful results observed in mice was because of the size difference of two animals, yet two dogs with different weights presented different FIX plasma levels. The studies with AAVes have demonstrated the increased and stable but not immediate production of FIX in animal models, because a high molecular weight head to tail concatemerization of AAV genome takes place before integration into host genome.

These successful experiments in animal models encouraged scientists for starting phase I clinical trials in the University of Pennsylvania and in the University of North Carolina.

H. Current Management of Hemophilia B: Genetic Counselling

Since FIX concentrates or fresh frozen plasma, used for the treatment of the disease, are prepared from a huge number of plasma pools, the therapy of the disease requires much effort and money. Moreover, there is always a risk of transmission of infectious diseases such as hepatitis B, AIDS or prion diseases. Recently, production of recombinant FIX eliminates this risk and makes the treatment easy but brings serious financial problems for patient families especially in a developing country.

The average cost of the treatment of a severely affected patient is 26000-30000 US dollars per year in Turkey. Therefore, identification of carriers and prenatal diagnosis is very important in order to reduce the familial transmission of the disease.

Once the mutation(s) in one patient is defined, accurate carrier identification could be performed for his/her relatives in familial cases. Prenatal diagnosis, also, could be given with 100% accuracy for such families.

Linkage analysis is a powerful tool used on carrier identification or prenatal diagnosis if the mutation of the patient in the family has not been identified, or for non-familial (sporadic) cases. In linkage analysis, the allele of the patient is traced through the haplotypes in the family. The individuals with the same haplotype as the patient(s) referred as carriers, if not germ-line or somatic mosaics, or affected persons.

II. PURPOSE

Hemophilia B is the third common coagulation disorder with an incidence of one in 30000 males. The disease is very heterogeneous at both clinical and molecular levels and is caused by the mutations in the FIX gene. The FIX gene is one of the extensively studied genes because of its relatively small size and the knowledge of its whole sequence. A large number of mutations affecting the gene have been found over the years since its identification in 1985. The accumulated data was collected in a database, which is updated annually. The most recent update has a patient entry of 1918, of which 689 have unique mutations. Delineation of causative mutations in such a large number of patients have contributed significantly to the understanding of the relationship between the structure of the gene and the function of its protein product; revealed new mechanisms that affect the human genome; and consequently, lead to accurate carrier identification and prenatal diagnosis of hemophilia B for which there is no cure yet, and the current therapy is difficult, expensive and risky. The ultimate cure for hemophilia B is gene therapy, and although many advances have been made in this field, there is still much to learn.

The number of hemophilia B patients in our country is expected to be about 1000. Previously, mutations in 10 Turkish hemophilia B patients have been identified. The purpose of this study is: (a) to increase the number of identified mutations to construct a mutation profile for the Turkish population and to compare it to the hemophilia B database; (b) to contribute to the knowledge of genotype-phenotype correlations in hemophilia B; (c) to construct haplotypes for hemophilia B patients to be used in association with the mutation and the phenotype of the patient; (d) to compile all of the obtained information in a national database; and (e) to analyze a hypervariable polymorphic site within the FIX gene to observe genotypic variation within or among populations.

III. MATERIALS

A. Blood and Chorionic Villus Samples

The peripheral blood samples from hemophilia B patients and their relatives, and chorionic villus samples were provided by the Department of Pediatric Hematology, Medical School, Ege University, İzmir; Department of Pediatric Hematology, Medical School, Hacettepe University, Ankara; Department of Molecular Pathology, Ankara University Children's Hospital, Ankara; Department of Pediatric Hematology, Medical School, Çukurova University, Adana; Department of Hematology, Cerrahpaşa Medical School, İstanbul University, İstanbul; Institute of Child Health, Division of Medical Genetics, Medical School; İstanbul University; and Department of Pediatrics, SSK Okmeydanı Hospital, İstanbul.

B. Buffers, Solutions, Enzymes, and Kits

All chemicals and solutions used in this study were from Merck (GERMANY) or Sigma (USA) unless stated otherwise in the text.

1. DNA Extraction from Peripheral Blood and Chorionic Villus Samples

Cell Lysis Buffer (for PBS) : 155 mM NH_4Cl, 10 mM $KHCO_3$,
0.1 mM $Na_2EDTA.2H_2O$ [pH 7.4]

Cell Lysis Buffer (for CVS)	:	400 mM NaCl, 100 mM Tris-HCl [pH 8.0], 5 mM $Na_2EDTA.2H_2O$ [pH 7.8], 0.2% SDS, 0.5 mg/ml Proteinase K
Nucleus Lysis Buffer	:	400 mM NaCl, 10 mM Tris-HCl [pH 8.0], 2 mM $Na_2EDTA.2H_2O$ [pH 7.8]
Sodium Dodecylsulphate	:	10 % SDS (w/v) in dH_2O [pH 7.2]
Proteinase K	:	20 mg/ml in dH_2O
Sodium Chloride	:	5 M in dH_2O
Ethanol	:	Absolute Ethanol, (Tekel, TÜRKİYE)
Isopropanol	:	Pure Isopropanol
TE Buffer	:	20 mM Tris-HCl [pH 8.0], 0.1 mM $Na_2EDTA.2H_2O$ [pH 8.0]

2. Polymerase Chain Reaction (PCR)

10 x $MgCl_2$ Free Buffer [pH 9.0]	:	500 mM KCl, 100mM Tris-HCl, 0.8% Nonidet P40, (MBI, LITHUANIA)
$MgCl_2$	:	25 mM in dH_2O, (MBI, LITHUANIA)
Deoxyribonucleotides	:	100 mM of dATP, dCTP, dGTP and dTTP, (Promega, USA)
Taq DNA Polymerase	:	5 U/µl, (MBI, LITHUANIA)

3. Haplotype Analysis

*Bam*H1 Restriction Enzyme	:	10 U/µl, (Promega, USA)
*Hha*I Restriction Enzyme	:	20 U/µl, (New England Biolabs, USA)
*Mnl*I Restriction Enzyme	:	5 U/µl, (New England Biolabs, USA)
*Taq*I Restriction Enzyme	:	10 U/µl, (Promega, USA)
*Xmn*I Restriction Enzyme	:	10 U/µl, (Promega, USA)
Buffer B	:	10 X Buffer for *Xmn*I, (Promega, USA)
Buffer E	:	10 X Buffer for *BamH*1 and *Taq*I, (Promega, USA)
NE Buffer 2	:	10 X Buffer for *Mnl*I, (New England Biolabs, USA)
NE Buffer 4	:	10 X Buffer for *Hha*I, (New England Biolabs, USA)

4. Agarose and Acrylamide Gel Electrophoresis

a. Electrophoresis Buffers.

10 X TBE Buffer [pH 8.3]	:	890 mM Tris-Base, 890 mM Boric Acid, 20 mM $Na_2EDTA.2H_2O$
20 x GTG Buffer	:	216 g/L Tris-Base, 72 g/L Taurine, 4 g/L $Na_2EDTA.2H_2O$

10 X Loading Buffer	:	2.5 mg/ml Bromophenol Blue (BPB), 1 % SDS in Glycerol
Stop Buffer I	:	50% Formamide, 7 M Urea, 2 mM $Na_2EDTA.2H_2O$, 0.05 % XC, 0.05 % BPB
Stop Buffer II	:	95 % Formamide, 20 mM $Na_2EDTA.2H_2O$, 0.05 % XC, 0.05 % BPB
Ethidium Bromide (EtBr)	:	10 mg/ml

b. Gel Systems.

1 or 2% Agarose Gel	:	1% or 2% (w/v) Agarose in 0.5 X TBE Buffer
40% Acrylamide Stock (19:1)	:	38% Acrylamide, 2% N,N'-Methylene Bisacrylamide
36% Acrylamide Stock (35:1)	:	35% Acrylamide, 1% N,N'-Methylene Bisacrylamide
8% Instagel Solution	:	8% Acrylamide Stock Solution (19:1), 8 M Urea, 1 X GTG Buffer
0.5 X MDE™ Gel Solution	:	0.5 X Hydrolink MDE™ Gel Solution, 0.5 X TBE Buffer [pH 8.3]
6% SSCP Gel Solution	:	6% Acrylamide Stock Solution (35:1), 0.5 X TBE Buffer [pH 8.3]

Ammonium Peroxodisulfate : 10 % APS (w/v) in dH_2O

TEMED : N',N',N',N'-Tetramethylethylenediamine

Silicone Reagent : 2% Dimethyldichloro Silane in Chloroform

c. DNA Size Markers.

ϕX174/*Hae*III Digest : 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp fragments, (Gibco BRL, USA).

λ/*Hin*dIII Digest : 23130, 9416, 6557, 4361, 2322, 2027, 564, and 125 bp fragments, (Promega, USA)

1 kb DNA Ladder : 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 506, 517, 396, 344, 298, 220, 201, 154, 134, 75 bp fragments (Gibco BRL, USA).

5. Dideoxy Fingerprinting and Genomic Amplification with Transcript Sequencing

5 X Transcription Buffer : 200 mM Tris [pH 7.5], 30 mM $MgCl_2$, 50 mM NaCl 10 mM Spermidine, (Promega, USA)

Spermidine : 40 mM in dH_2O

Dithiothreitol : 100 mM DTT, (Promega, USA)

Ribonucleotides	:	100 mM of dATP, dCTP, dGTP, and dUTP, (Boehringer-Mannheim, GERMANY)
T7 RNA Polymerase	:	10-20 U/µl, (Promega, USA)
10 X Polynucleotide Kinase Buffer	:	500 mM Tris, 100 mM $MgCl_2$, 50 mM DTT, 1 mM Spermidine, (Promega, USA)
T4 Polynucleotide Kinase	:	10 U/µl, (Promega, USA)
Reverse Transcriptase Buffer	:	24 mM Tris [pH 8.3], 16 mM $MgCl_2$, 8 mM DTT, 0.8 mM dATP, 0.4 mM dCTP, 0.8 mM dGTP, 1.2 mM dTTP
Annealing Buffer	:	250 mM KCl, 10 mM Tris-HCl
Actinomycin D	:	1 mg/ml in dH_2O
Dideoxyribonucleotides	:	10 mM of ddATP, ddCTP, ddGTP, and ddTTP, (Boehringer-Mannheim, GERMANY)
AMV Reverse Transcriptase	:	5-10 U/µl, (Promega, USA)

6. QIAquick-spin PCR Purification Kit (QIAGEN, USA)

Buffer PB	:	Chaotropic salt solution of unknown content
Buffer PE	:	Alcohol mixture of unknown content
Buffer EB	:	10 mM Tris-HCl

7. Omnibase™ DNA Cycle Sequencing Kit (Promega, USA)

DNA Sequencing 5 X Buffer	:	250 mM Tris-HCl [pH 9.0], 10 mM $MgCl_2$
d/ddA Nucleotide Mix	:	20 μM of dATP, dCTP, dTTP and 7-deaza dGTP, including 0.2 μM ddATP
d/ddC Nucleotide Mix	:	20 μM of dATP, dCTP, dTTP and 7-deaza dGTP, including 0.25 μM ddCTP
d/ddG Nucleotide Mix	:	20 μM of dATP, dCTP, dTTP and 7-deaza dGTP, including 0.3 μM ddGTP
d/ddT Nucleotide Mix	:	20 μM of dATP, dCTP, dTTP and 7-deaza dGTP, including 0.25 μM ddTTP
Sequencing Enzyme Mix	:	OmniBase™ DNA polymerase and Tth pyrophospatase mix (10U/μl)
T4 Polynucleotide Kinase	:	10 U/μl
10 x Polynucleotide Kinase Buffer	:	500 mM Tris-HCl [pH 7.5], 100 mM $MgCl_2$, 50 mM DTT, 1 mM Spermidine
pGEM-3Zf(+) Control DNA	:	200 ng/μl
pUC/M13 Forward Primer	:	10 μg/ml 5'-d(CGCCAGGGTTTTCCCAGTCACGAC)-3'
Stop Solution	:	10 mM NaOH, 95% formamide, 0.05 % BPB (v/v), 0.05 % XC (v/v)

8. Radioactive Labeling and Autoradiography

γ-[^{32}P]-dATP	:	>5000 Ci/mmol Radionucleotide, (Amersham, UK)
α-[^{35}S]-dATP	:	>1000 Ci/mmol Radionucleotide, (Amersham, UK)
Developer and Fixer	:	KODAK, USA

C. Others

Instant Films	:	Polaroid 667, (POLAROID, UK)
3MM Chromatography Paper	:	46 x 57 cm, (Whatman, UK)
X-Ray Films	:	X-Omat AR-5, (KODAK, USA)

D. Oligonucleotide Primers

The FIX specific PCR primers used in mutation screening studies were either provided by Dr. Steve S. Sommer from the Beckman Research Institute, City of Hope, USA, or purchased from Iontek (TÜRKİYE). The PCR primers that were used for haplotype analysis were from Boehringer-Mannheim. Sequences of the primers used in the mutation screening and identification, and haplotype analysis of the FIX gene are given in Tables III.1-5.

TABLE III.1. Sequences of the PCR primers used in mutation analysis. F: Forward primer; R: Reverse primer.

Exon	Primer Sequence (5'→3')	Location	PCR Product Size (bp)
A	F-*AGGCAAGCATACTCAATG R- AGCCACTATGCCTAT	157 -239	425
B/C	F-*GACTTTCTTAAGAGATGT R- GATCTTTCTGAGTCCT	6247 6818	600
D	F-*AATCAGACTCCCATCC R- ATGATACACCAATATTGC	10299 10591	320
E	F-*CCCATACATGAGTCAG R- AAGCAGATTCAAGTAGG	17585 17894	338
F	F-*TGCCAATGAGAAATATCAGG R- ATACTTCTCACATCCC	20675 20316	388
G	F-*ATTCCTCAGATTTGCC R- TATGACCCTTCTGCC	29784 30280	525
H	F-*CCCAAGTAGTCACTTAG R- TATGAAATTCTCCCCTG	30747 31515	797

*29 bp T7 phage promoter sequence: GGTACCTAATACGACTCACTATAGGGAGA

TABLE III.2. Sequences of the nested primers used in dideoxy fingerprinting and sequencing reactions. US: Upstream; DS: Downstream.

Exon	Primer Sequence (5'→3')		Location
A	A1	CACAATCTGCTAGCAAA	9 (DS)
	A2	TCTCTTCACTTGTCC	-121 (DS)
	A3	ACTCAATGTATTTTAAAAAAG	147 (US)
B	B2	TCTCAAGGTTCCCTTG	6420 (US)
	B1	CTATTCTATGCTCTGCA	6535 (US)
C	C1	CCCACATAATTCTCATAT	6759 (US)
D	D2	GTCATCCTTGCAACTG	10447 (US)
	D1	AACTTGTTTCAGAGGG	10560 (US)
E	E2	GAGCAAACCACCTTGT	17744 (US)
	E1	TGTAGGTTTGTTAAAATGC	17866 (US)
F	F2	CATAGTCCACATCAGG	20446 (US)
	F1	AGCCTCAGTCTCCCA	20638 (US)
G	G1	TATGGTGTACCAATCATA	30219 (US)
H	H1	TGAGGAAGATGTTCGTG	31022 (US)
	H2	ATGTTGTTATAGATGG	31164 (US)
	H3-2	GCCTTTCATTGCACAC	31300 (US)
	H3-1	TAGTGAGAGGCCCTGTTA	31429 (US)
	H4	TACACGAACATCTTCCTC	31004 (DS)

TABLE III.3. Sequences of the PCR and nested primers used in the analysis of patients with gross rearrangements. D: Downstream; U: Upstream; I: Intron; E. Exon.

Name	Primer Sequence (5'→3')
F9-5'UTR(-2425)-19D	AAAAGAGCCCGCATCGCCA
F9-5'UTR(-882)-17D	CAATGGCACAAAGAATA
F9-(T7-23)-5'UTR(-238)-38D	(T7-23)-AGCCACTATGCCTAT
F9-5'UTR(-239)17D	GTAGCCACTATGCCTAT
F9-I1(845)-17D	CTAATGCAACATATATC
F9-(T7-23)-I1(1783)-38D	(T7-23)- GCCAGGTACTGTGTC
F9-(T7-23)-I1(2780)-40D	(T7-23)-AGTTGGAAAGATAATCT
F9-(T7-23)-I1(4032)-39D	(T7-23)-TTCCATGGATACTGAG
F9-(T7-23)-I1(4132)-37D	(T7-23)-TGGAGGCTGTGAGG
F9-I1(5337)-17D	GTCCATCATTGACCAAA
F9-I1(-341)-20U	GCAATGTATGAGTGGTCCAG
F9-(Sp6-29)-E1(155)-46U	(Sp6-29)-GCAAGCATACTCAATGT
F9-I1(1093)-15U	ATCATGTCTGGAGTG
F9-(Sp6-23)-I1(2283)-40U	(Sp6-23)-ATTTCATTAGCGTGTTG
F9-(Sp6-23)-I1(3066)-38U	(Sp6-23)-GGCATGTAGGGCACA
F9-(Sp6-23)-I1(5129)-41U	(Sp6-23)-AGGTAAGTACACTCTA---------
F9-(Sp6-23)-I1(5229)-39U	(Sp6-23)-GTACAGTCACATGCTG
F9-I1(5719)-17U	TACATCTACCTAGTCTG

T7-23 : TAATACGACTCACTATAGGGAGA

Sp6-23 : ATTTAGGTGACACTATAGAATAC

Sp6-29 : GGTACCATTTAGGTGACACTATAGAATAC

TABLE III.4. Sequences of the PCR primers used in haplotype analysis. F: Forward primer; R: Reverse primer.

Marker	Primer Sequence (5'→3')	Location	PCR Product Size (bp)
*Bam*HI	F- GAAGTTTGACCTAAACATCATAC	-708	524
	R- TTGAGTCTGAAACAGGAAGTGA	-184	
*Dde*I	F-ATGTGGTCCATCATTGACCA	5332	380-430
	R-ACCTAGTCTGAAGAGACACT	5711	
*Taq*I	F- TATAATGGGAATTCTCCACAT	11015	299
	R- AGTAGAAAGTGAATTCCTCA	11314	
*Xmn*I	F-CAGAGACTGCTGATTGACTT	7014	220
	R-ACAGCCAGATAAAGCCTCCA	7234	
*Mnl*I	F-GATTTGAAAACTGTCCATGAAAATAAC	20166	406
	R-AAAGTACCTGCCAAGGGAATTGACCTG	20572	
*Hha*I	F-ACAGGCACCTGCCATCACTT	8 kb to	230
	R-AGATTTCAAGCTACCAACAT	3'UTR	

TABLE III.5. Sequences of the primers used in the analysis of the intron 1cRY(i) region. DS: Downstream; F: Forward primer; R: Reverse primer.

Primer	Primer Sequence (5'→3')	Location
PCR	F-ATGTGGTCCATCATTGACCA	5332
	R-ACCTAGTCTGAAGAGACACT	5711
Sequencing	AATGTCATTGTGCAGCA	5353 (DS)

E. Equipment

Autoclaves	:	Model MAC-601, (Eyela, JAPAN); MIDAS 55, (Prior Clave, UK)
Balances	:	Electronic Balance VA124 , (Gec Avery, UK); Electronic Balance CC181, (Gec Avery, UK)
Centrifuges	:	SORVALL RC-5B Refrigirated Superspeed Centrifuge, (Dupont, USA); Centrifuge 5415 C, (Eppendorf, GERMANY)
Refrigerators	:	$+4^0$C, (Arçelik, TÜRKİYE);
Deepfreezers	:	-70^0C Ultralow, (Sanyo, JAPAN); -20^0C, (Bosch, GERMANY)
Documentation Apparatus	:	Direct Screen Instant Camera, (Polaroid, UK); BioDoc Controller, (Biometra, GERMANY); Video Monitor WVBM900 (Biometra, GERMANY); Video Graphic Printer UP890CE, (Sony, JAPAN)
Electrophoretic Equipment	:	HORIZON™ 58 Horizontal Gel Electrophoresis Systems, (Gibco BRL, USA); Model H5 Horizontal System for Submerged Gel Electrophoresis, (Gibco BRL, USA); Model S2, (Gibco BRL, USA); Base Ace™ Jr., (Stratagene, USA)
Gel Dryer	:	Biometra, GERMANY

Incubator	:	NUVE EN400
Power Supplies	:	ECPS 3000 / 150 Constant Power Supply CESA 1110, (Pharmacia, SWEDEN); EPS 250, (CBS Scientific Company, USA)
Spectrophotometer	:	Lambda 3 UV/VIS, (PEC, USA); CE 5502 Scanning Double Beam 5000 Series, (CECIL Elegant Technology, UK)
Thermo-Cyclers	:	PTC-200, (MJ Research, USA); Progene, (Techne, UK); UNO Thermoblock, (Biometra, GERMANY)
Transilluminator	:	Dual Intensity UVP Consort
Water Baths	:	Thermomix, BU, (Braun, GERMANY) Type 2048, DVE (Kottermann, GERMANY)

IV. METHODS

A. Extraction of Genomic DNA

1. Peripheral Blood Samples

Ten ml of peripheral blood samples were lysed in 30 ml ice-cold cell lysis buffer at 4°C for 15 minutes. The leukocyte nuclei were pelleted by centrifugation at 5000 rpm for 10 min. After centrifugation, the supernatant was discarded and the pellet was washed in 10 ml of cell lysis buffer, resuspended and centrifugation step was repeated. Then, the nuclear pellet was resuspended in 3 ml of ice-cold nucleus lysis buffer and incubated with 30 µl proteinase K and 50 µl SDS at 56°C for 3 hours or at 37°C overnight. Following the incubation, the protein debris was salted out by adding 5 ml of saturated NaCl and 5 ml of water. The sample was centrifuged at 10000 rpm for 20 minutes at room temperature. The DNA was precipitated with two volumes of absolute ethanol. DNA was fished out, dried and dissolved in appropriate amount of TE buffer [121].

2. Chorionic Villus Samples

The sample was centrifuged to collect the cells from the medium. The pellet was resuspended gently in 1 ml of cell lysis buffer and incubated at 37°C overnight. Then, the DNA was precipitated by adding two volumes of isopropanol, and was dissolved in appropriate amount of TE buffer.

3. Determination of DNA Quantity

The concentration and the purity of the homogeneously dissolved DNA samples were detected spectrophotometrically. The DNA sample was diluted in distilled water and the optical densities were read at 260 nm (A_{260}) and at 280 nm (A_{280}). The concentration of the dissolved DNA was calculated by using the formula:

$$\text{Concentration } (\mu g/ml) = \text{Dilution factor} \times A_{260} \times 50\ \mu g/ml$$

The purity of DNA was estimated by the ratio of A_{260} / A_{280}. Samples having a ratio of 1.8 ($\pm$ 0.1) were considered pure.

4. Analysis of DNA Quality

One gram agarose was boiled in 100 ml of 0.5 X TBE buffer to prepare 1 per cent agarose (w/v). The solution was cooled to 56°C and 5 µl ethidium bromide was added. Twenty-five mililiters of the solution was poured into gel casting tray and let to polymerize. One µl of DNA sample was mixed with 1 X loading buffer in a total of 10 µl volume and applied into the polymerized gel. The gel was electrophoresed in 0.5 X TBE buffer at 150 V and DNA was visualized using UV light transilluminator. Thus, the quality of the genomic DNA was checked before proceeding further.

B. Mutational Analysis of the Factor IX Gene

1. Dideoxy Fingerprinting (ddF)

Dideoxy fingerprinting is a rapid mutation detection method [95] that involves the amplification of the regions of likely functional significance of the FIX gene with T7 or Sp6 promoter sequence tagged PCR primers. The products are then *in vitro* transcribed and used in a single sequencing reaction with one of the dideoxynucleotides (ddNTPs), which were chosen according to the base composition of the amplified region, and analysed on a non-denaturing polyacrylamide gel system. The use of nested primers for sequencing reactions enhances the specificity. The regions that are suspected to have a change are then sequenced to delineate causative mutations.

a. PCR. Eight exons, exon-intron boundaries, splice sites, and promoter, and a part of 3' UTR were amplified in seven PCRs using the primers given in Table III.1.

The amplification reactions were performed in a total volume of 25 μl, containing 1 X $MgCl_2$-free reaction buffer, 1.5 or 2.5 mM $MgCl_2$, 200 μM dNTP, 2.5 pmoles of each primer, 0.5 U *Taq* polymerase and 250-300 ng genomic DNA. Thirty-cycle PCR program included a denaturation step of 94°C for 1 minute, an annealing step of 50 °C for 2 minutes, and an extension step of 72°C for 3 minutes. Following these 30 cycles, the products were further extended for another 10 minutes at 72°C.

After amplification, 5 μl of each reaction was mixed with 5 μl of 1 X loading buffer and run on 2 per cent agarose gel in order to check the amount and the

specificity of amplification. If the results were satisfactory, products were kept at -20°C until use.

b. Transcription. Transcription reactions were carried out in a total volume of 20 µl, including 1 X transcription buffer, 500 µM of ribonucleotides, 10 mM dithiothreitol (DTT), 10 U T7 RNA polymerase, and 3 µl PCR product from the previous section. The mixture was incubated at 37°C for 1.5 hours.

One µl of each transcript was checked on a 2 per cent agarose gel and then frozen at -20°C until use.

c. Sequencing Reaction. A 100 ng sample of nested sequencing primer, given in Table III.2., was end-labeled in a reaction volume of 13 µl, containing 1 X T4 polynucleotide kinase buffer, 100 µCi [γ-^{32}P]-dATP, and 10 U of T4 polynucleotide kinase for 30 minutes at 37°C. The excess enzyme was inactivated at 65°C for 5 minutes, and 7 µl of dH_2O was added for a final 5 ng/µl concentration of the primer.

For sequencing, 0.5-2 µl of the transcript and 1 µl of end-labeled sequencing primer were added to 10 µl of annealing buffer. The samples were heated to 80°C for 3 minutes and then annealed for 30 minutes at 45°C. Meanwhile, a mixture of 15 µl actinomycin D and 135 µl of reverse transcriptase buffer was prepared. One µl of appropriate dideoxy nucleotide (ddNTP) and 1 U AMV reverse transcriptase were added to 3.3 µl of the above mixture for 1 µl of each transcript. The mix was incubated at 55°C for 45 minutes and the reaction was stopped by adding 10 µl of stop buffer I. Samples were kept at -20°C until electrophoresis.

d. Gel Preparation and Electrophoresis. The glass plates were cleaned first with water and detergent, then with distilled water, and with alcohol at last. The small plate was siliconized at inside face (this step was only performed before the first use of the plates). Then, 0.4 mm thick spacers were placed between two glass plates on two sides. The plate-spacer sandwich was fixed by clamps on both sides. The sandwich was placed horizontally and balanced for a flat surface. The 2 X concentrate MDE gel solution was diluted to a 0.5 X concentration through mixing with 3 ml of 10 X TBE buffer and 42 ml dH_20 in a total volume of 60 ml. Alternatively, 6 per cent SSCP gel solution, prepared by mixing 10 ml of 36 per cent acrylamide stock (35:1) with 3 ml of 10 X TBE buffer in a total volume of 60 ml, was used when MDE gel was not available. Polymerization of either mixture was initiated by adding 600 µl 10 per cent APS and 60 µl TEMED. The gel solution was applied into the space between two glass plates using a 60 ml-syringe. A 0.2 mm thick-square well forming comb was inserted between the plates. The gel was covered with plastic wrap to prevent drying-up and left overnight for polymerization.

Electrophoresis was carried out in 0.5 X TBE buffer. The comb was taken out and the gel was pre-electrophoresed for 15 minutes at 20 W constant power. The samples were denatured at 95°C for 3 minutes and chilled on ice. The slots were flushed with buffer carefully to clean impurities and 3 µl of each sample was loaded on each lane. Electrophoresis was performed at a 20 W constant power at room temperature until bromophenol blue (BPB) leaves the gel.

e. Autoradiography. After electrophoresis was stopped, the plates were separated and a 3 MM Whatmann paper, cut to the size of the gel, was laid on the gel. The paper and the gel were removed together, and the gel was covered with plastic wrap. After drying the gel for 30 minutes in a gel dryer, plastic wrap was removed. The dry gel was exposed to an X-ray film, with an intensifying screen, in an exposure casette at -70°C for overnight. The film was developed manually

for 3 minutes, rinsed with tap water, and fixed for another 3 minutes. Then, it was rinsed with tap water again and dried in an oven for a few minutes.

2. Direct DNA Sequencing

a. Genomic Amplification with Transcript Sequencing (GAWTS). The PCR, transcription, end-labeling and sequencing reactions were performed as described in sections IV.B.1.a., IV.B.1.b., and IV.B.1.c., respectively. The only difference was the use of all four ddNTPs instead of one and analyzing the reactions in denaturing polyacrylamide gel systems [122].

b. OmniBase™ DNA Cycle Sequencing Kit. Initially, PCR mixes (prepared as described in section IV.B.1.a.) were purified using QIAquick PCR purification kit according to the protocol described by the manufacturer. Fifty µl of the PCR product was mixed with 5 volumes of PB buffer on Vortex and put into a QIAquick spin column. The PB buffer contains a chaotropic salt (pH≤7.5) which allows the efficient binding of single or double stranded DNA as small as 100 bp and removes approximately 100 per cent of primers up to 40 nucleotides. The column, containing a silica-gel membrane, was placed in a 2 ml collection tube to adsorb the DNA in the presence of a high concentration of chaotropic salts. The sample was centrifuged in this column at 13,000 rpm in a microcentrifuge for 30-60 sec. Flow-through was discarded and QIAquick column was placed back into the same tube. The column was then washed with 750 µl of absolute ethanol-containing PE buffer by centrifuging as above. Flow-through was rediscarded after each centrifugation. Residual wash buffer was removed through the column completely by another centrifugation step. To elute DNA bound onto the membrane, QIAquick column was placed in a clean 1.5 ml microfuge tube and 50 µl of either high pH-deionized water or elution buffer was added to the center of the QIAquick column and centrifuged for 1 min. The quality of purification was checked on a 2 per cent agarose gel.

For each set of sequencing reactions, four tubes were labelled as A, T, G, and C. Then, 2 µl of each d/ddNTP mix was aliquoted into appropriate tubes and kept on ice until needed. A mixture was prepared, containing 1.5 X DNA sequencing buffer, 10 µCi of [α-^{35}S]-dATP, 3 pmol of nested sequencing primer, 120 fmol of purified template DNA, and 10 U of OmniBase™ sequencing enzyme mix in a total volume of 17 µl. Then, 4 µl of this mixture was aliquoted into each d/ddNTP mix tubes and the mixes were overlaid with a drop of mineral oil. The reaction tubes were then placed in a thermal cycler that had been preheated to 95°C and cycling program was started. One of two programs was chosen according to the length and the GC content of the sequencing primer used.

When the primer length was shorter than 24 bases, or GC-content was lower than 50 per cent, Program 1 was used, whereas if the primer length was equal or larger than 24 bases, or GC-content was equal or more than 50 per cent, the Program 2 was used. The mentioned programs are given in Table IV.1.

TABLE IV.1. The cycling programs used in sequencing reactions performed with OmniBase™ DNA Cycle Sequencing Kit.

Program 1			**Program 2**		
Denaturation	95°C→2 min		Denaturation	95°C→2 min	
Denaturation	95°C→30 sec	30 cycles	Denaturation	95°C→30 sec	30 cycles
Annealing	42°C→30 sec		Annealing/ Extension	70°C→30 sec	
Extension	70°C→1 min				
Soak	4°C		Soak	4°C	

The products were put on ice immediately after the PCR amplification and 3 µl of stop solution was added to each tube. The products were stored at –20°C until use.

c. Gel Preparation and Electrophoresis. The glass plates were prepared as described in section IV.B.1.d., and the gel solution, containing 60 ml of 8 per cent instagel, 300 µl of 10 per cent APS, and 30 µl of TEMED, was applied between the plates by a 60 ml syringe. The flat edge of the shark-tooth comb was placed into the gel and the gel was let to polymerize at room temperature for at least three hours. If the gel was left overnight, the comb and the bottom of the gel were covered with plastic wrap to prevent drying out.

After polymerization, the comb was taken out and the gel was pre-electrophoresed in 1 X GTG buffer at a 80 W constant power for half an hour. The big slot, created with the removal of comb, was flushed with buffer to remove excess urea and the comb was re-inserted carefully so that the teeth just made a contact with the gel surface. The slots were flushed again. The sequencing reactions, either from section IV.B.2.a. or IV.B.2.b., were denatured at 95°C for 3 minutes and chilled on ice prior to loading. Three µl of each product was loaded into each slot and the gel was ran at 80 W constant power until the desired separation was achieved.

d. Autoradiography. After the electrophoresis was terminated, the glass plates were cooled down under tap water and carefully separated. A 3MM chromatography paper of appropriate size was placed on top of the gel and the gel was removed. The gel was dried in a gel dryer and exposed to an X-ray film in exposure cassette at room temperature for 1 to 3 days. The X-ray film was developed as described in section IV.B.1.e.

C. Haplotype Analysis

Haplotypes of the hemophilia B patients and their relatives were constructed by analyzing six polymorphic marker of the FIX gene; *Bam*HI, *Dde*I, *Xmn*I, *Taq*I, *Mnl*I, and *Hha*I. Five of the six polymorphic markers were analyzed by digestion of the PCR products with appropriate restriction enzymes. *Dde*I, which was the highly polymorphic VNTR region of the gene, was studied only by PCR.

1. PCR

The amplification reactions for all markers were performed in a total volume of 25 μl, containing 1 X $MgCl_2$-free buffer, 200 μM of each dNTPs, 0.5 U of *Taq* polymerase, 250-300 ng of genomic DNA, and varying amounts of $MgCl_2$ and specific primers. Table IV.2. shows the primer amounts and $MgCl_2$ concentrations used for each specific region.

TABLE IV.2. The primer and $MgCl_2$ concentrations used in the PCRs of the polymorphic marker regions.

Polymorphic Marker	Primer Concentration (pmoles)	$MgCl_2$ Concentration (mM)
*Bam*HI	6.25	2
*Dde*I	2.5	2
*Xmn*I	2.5	2
*Taq*I	2.5	3
*Mnl*I	12.5	2
*Hha*I	2.5	3

The samples were initially denatured at 94°C for 3 minutes. Then, a thirty-cycle PCR program including a denaturation step of 94°C for 30 seconds, and an annealing/extension step of 60°C for 2.30 minutes, was introduced. Following the 30 cycles, the products were further extended for another 10 minutes at 60°C.

The PCR products were checked on 2 per cent agarose gels through running in 0.5 X TBE buffer at 150 V. The PCR products of the *Dde*I region were run on 2 per cent agarose gels, at 100 V for about 30 minutes in order to have a fine separation of bands differing 24 or 26 bp or 50 bp from each other in length.

2. Restriction Enzyme Analysis

The PCR products of *Bam*HI, *Xmn*I, *Taq*I, *Mnl*I, and *Hha*I were subjected to restriction enzyme analysis in order to see whether they have the cutting site for the appropriate restriction enzymes. Five µl of each PCR product was mixed with 1 X reaction buffer and 1 U of appropriate restriction enzyme in a total volume of 20 µl for each enzyme and different fragments were created following overnight incubation at specific digestion temperatures (Table IV.3.).

TABLE IV.3. The digestion conditions of restriction enzymes used in haplotype analysis, and the lengths of the digestion products.

Enzyme	Digestion Temperature	Fragment Sizes after Digestion (bp)	
		(-) Allele	(+) Allele
*Bam*HI	37°C	356	216+140
*Xmn*I	37°C	222	154+68
*Taq*I	65°C	299	190+109
*Mnl*I	37°C	126+279	126+120+159
*Hha*I	37°C	230	150+80

The fragments were analyzed on 2 per cent agarose gels and the fragment sizes were determined by comparison with DNA molecular weight markers. An allele bearing a cutting site for the restriction enzyme was called as (+), whereas an allele that does not have the cutting site for that enzyme was called (-) allele. A DNA sample of known haplotype was included in each reaction to compare the results of digestions.

D. Analysis of the Intron 1 cRY(i) Region

1. PCR

The region bearing the hypervariable repeats were amplified in a 25 µl reaction, containing 1 X $MgCl_2$-free buffer, 200 µM of each dNTPs, 0.5 U of *Taq* polymerase, 250-300 ng of genomic DNA, 3 mM $MgCl_2$ and 2.5 pmoles of PCR primers.

The quality and quantity of the reactions were then analyzed on 2 per cent agarose gels, in the presence of a suitable DNA molecular weight marker, at 100 V for about 30 minutes.

2. Sequencing Analysis

The samples of high quality and quantity were subjected to DNA sequencing analysis as described in sections IV.B.2.b., IV.B.2.c., and IV.B.2.d.

V. RESULTS

In the framework of this thesis, mutational analysis of functionally significant regions of the FIX gene from 47 individuals (by either ddF or direct DNA sequencing) have been carried out; haplotypes for the patients have been constructed utilizing the polymorphic regions of the gene; the hypervariable cRY(i) region in intron 1 has been analyzed by direct sequencing; and the recent strategy for molecular diagnosis of hemophilia B has been established.

A. Mutational Analysis of the Factor IX Gene

1. Dideoxy Fingerprinting

The coding regions, exon-intron boundaries and promoter regions of the FIX gene, which makes a total of 2.2 kb, were analyzed by ddF in 38 individuals with hemophilia B. These samples were collected between the years 1994-1998 from various university hospitals. Figures V.1.-V.4. show the examples of ddF reactions for all analyzed regions of FIX genes from different individuals. A total of 24 aberrant patterns were observed in various ddF reactions of 23 individuals (Table V.1.). Exon A of two individuals did not amplify by PCR and there were no aberrant patterns observed in the remaining exons. In 13 individuals, aberrant patterns were not observed by ddF. The hemophilia B diagnosis was repeated for only six individuals by the clinical departments and they were not confirmed.

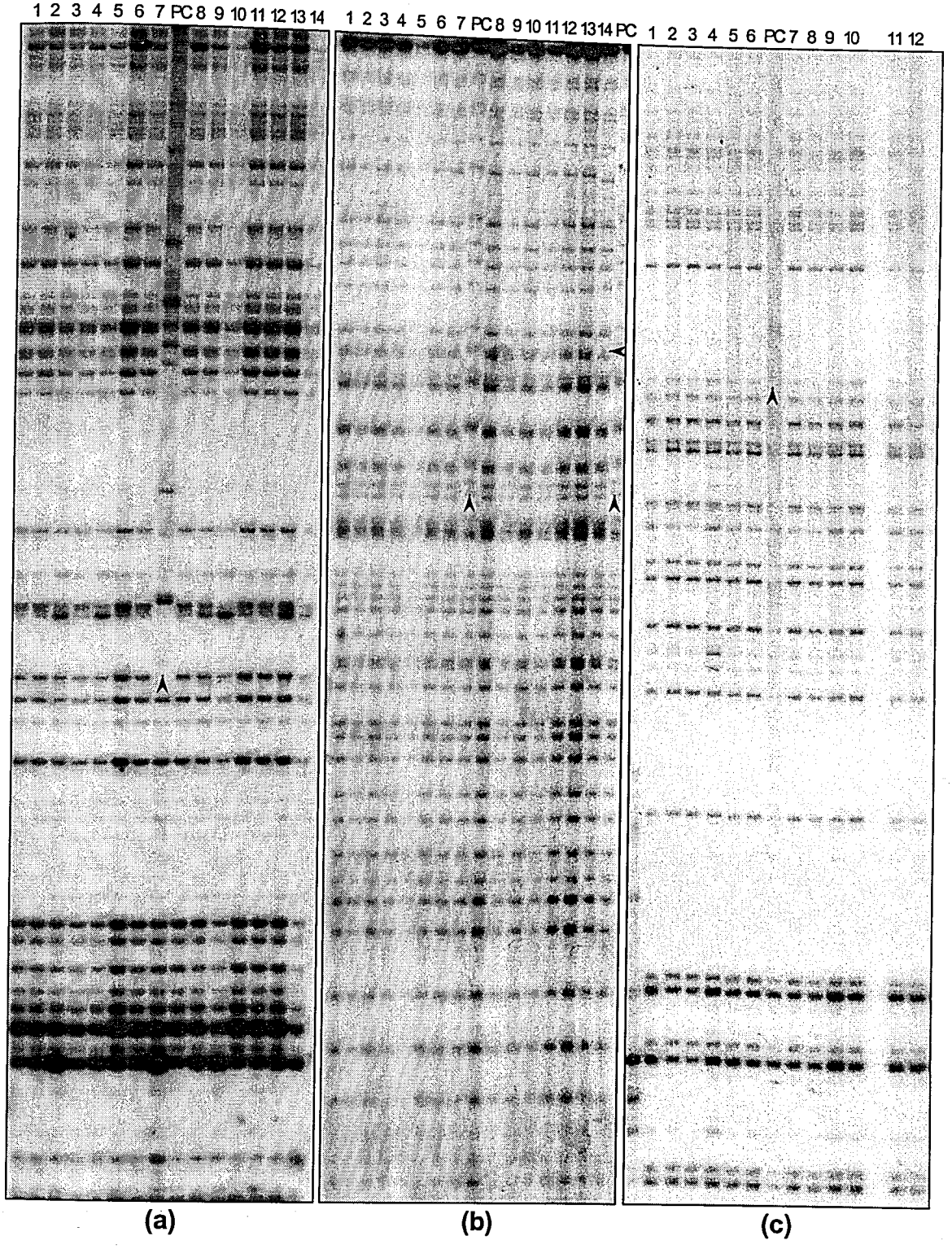


FIGURE V.1. Dideoxy fingerprinting analysis of exon A **(a)**, exon B **(b)**, and exon C **(c)** of the FIX gene on a 0.5 X MDE gel from various patients. The arrows show the starting point of the shift observed in DNA fragments in positive control (PC) samples and in samples with unknown mutations. Lane 14 of exon B belongs to HBT 150.

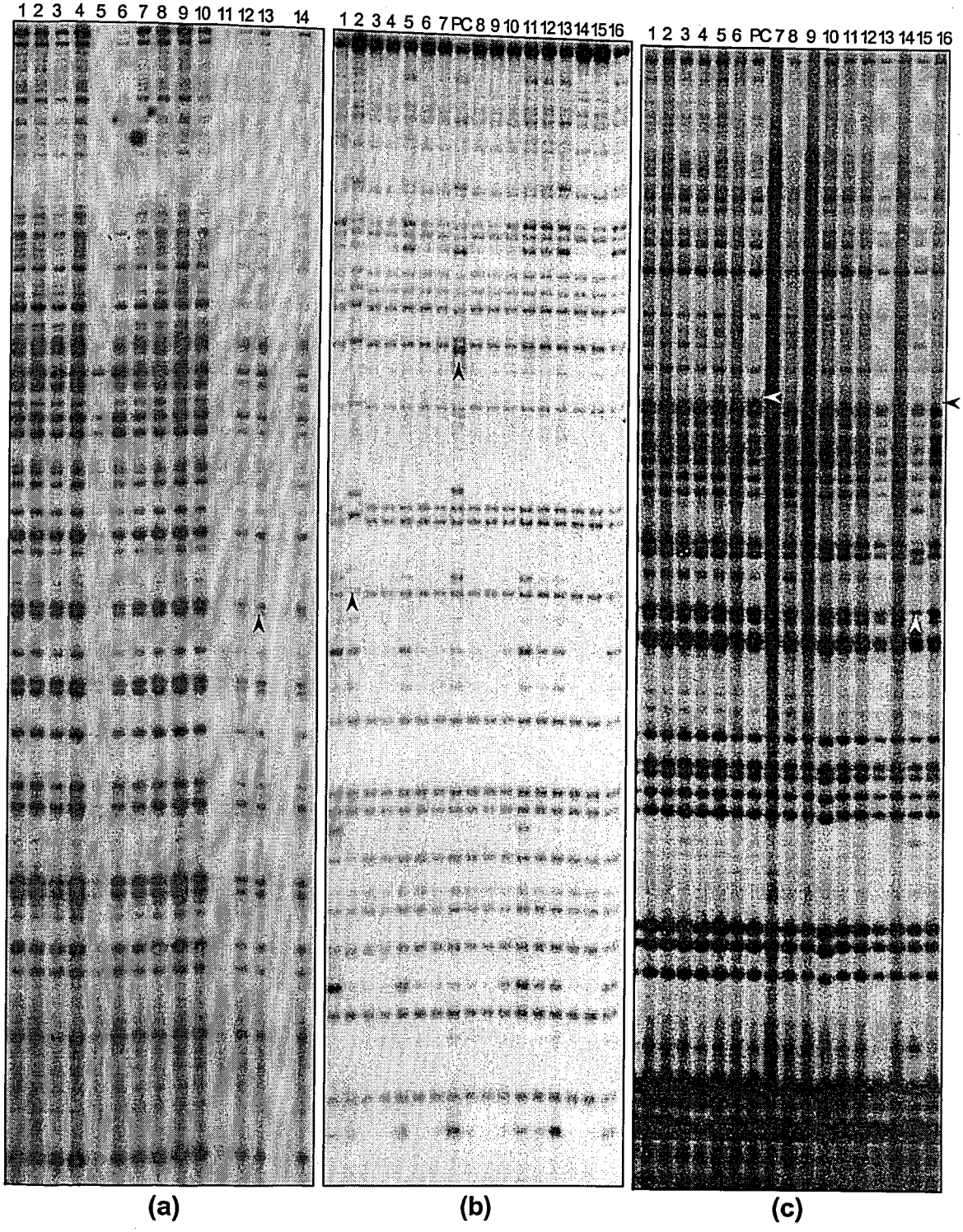


FIGURE V.2. Dideoxy fingerprinting analysis of exon D **(a)**, exon E **(b)**, and exon F **(c)** of the FIX gene on a 0.5 X MDE gel from various patients. The arrows show the starting point of the shift observed in DNA fragments in positive control (PC) samples and in samples with unknown mutations. Lanes 13 of exon D, 2 of exon E, and 15 and 16 of exon F belong to HBT 123, HBT 87, HBT 153 and HBT 154, respectively.

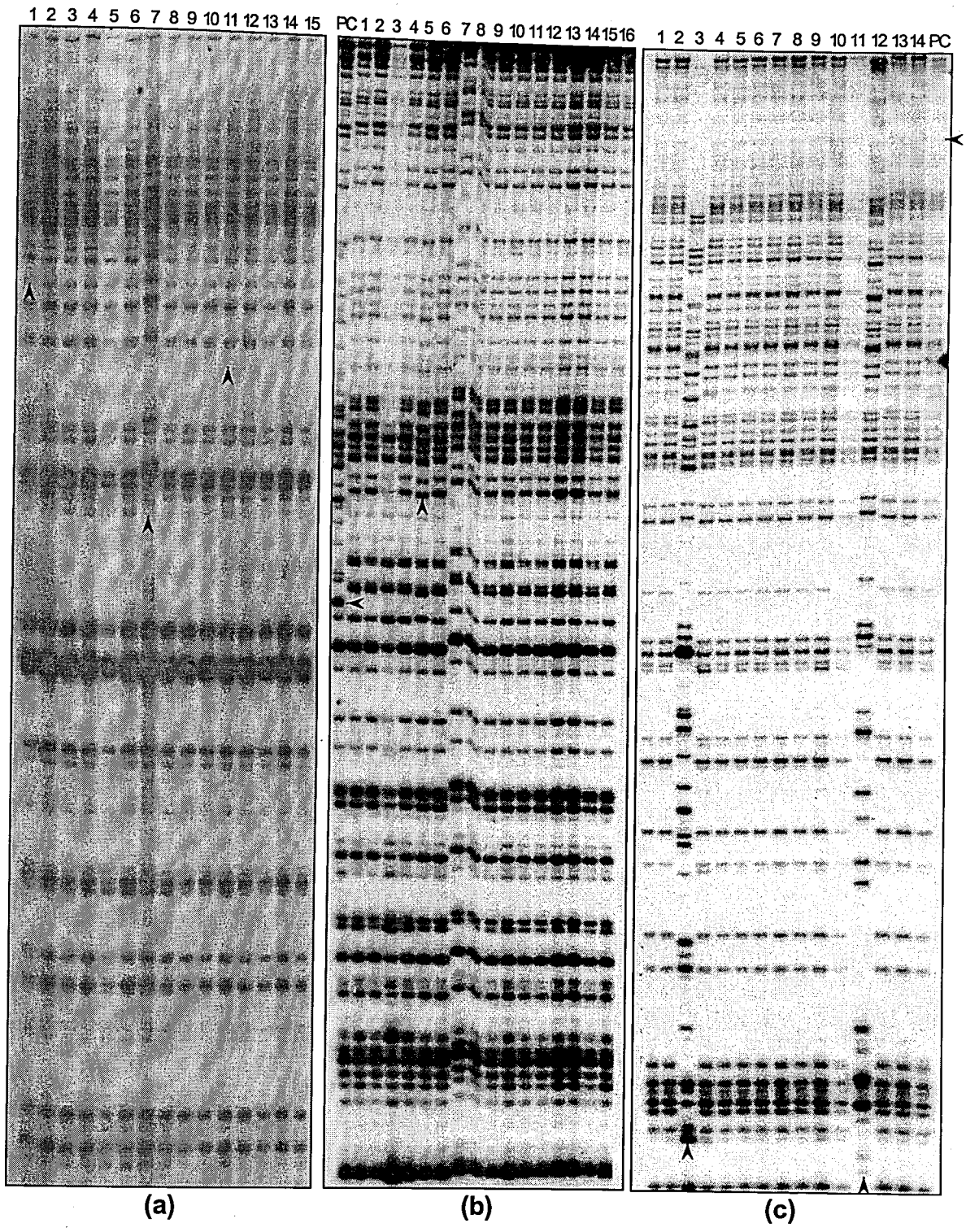


FIGURE V.3. Dideoxy fingerprinting analysis of exon G **(a)**, exon H (H1) **(b)**, and exon H (H2) **(c)** of the FIX gene on a 0.5 X MDE gel from various patients. The arrows show the starting point of the shift observed in DNA fragments in positive control (PC) samples and in samples with unknown mutations. Lanes 1 and 7 of exon G, 5 of exon H1, and 3 and 12 of exon H2 belong to HBT 158, HBT 163, HBT 56, HBT 62 and HBT 93, respectively.

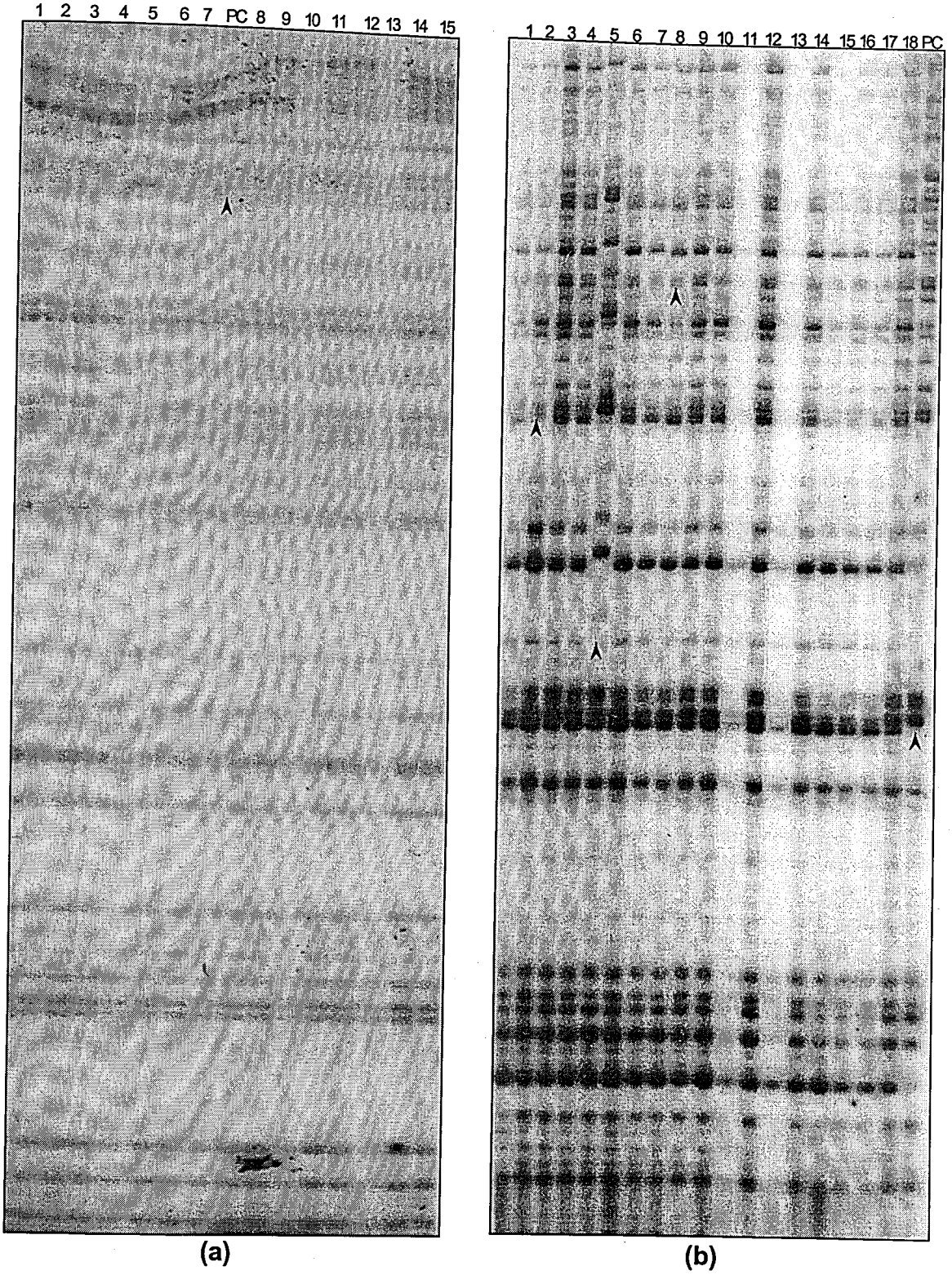


FIGURE V.4. Dideoxy fingerprinting analysis of exon H (H3-1) **(a)**, and exon H (H3-2) **(b)** of the FIX gene on a 0.5 X MDE gel from various patients. The arrows show the starting point of the shift observed in DNA fragments in positive control (PC) samples and in samples with unknown mutations. Lanes 2, 5, and 8 of exon H3-2, belong to HBT 155, HBT 157 and HBT 161, respectively.

TABLE V.1. The segments of the FIX gene screened by dideoxy fingerprinting in 38 individuals. Shaded boxes show the regions in which an abnormal pattern is observed. (-) sign means the corresponding region was not analyzed by ddF, (+/+) sign means the corresponding region was analyzed by ddF and the reaction quality was good, and (+/-) sign means the corresponding region was analyzed by ddF but the reaction quality was poor. The last six shaded rows show the individuals whose hemophilia B diagnosis were not confirmed.

	DNA No	Promoter & A1	B1	C	D1	E1	F1	G	H1	H2	H3-1	H3-2
1	2 HBT 48	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
2	11 HBT 87	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
3	14 HBT 62	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
4	16 HBT 118	+/+	+/+	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
5	18 HBT 56	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
6	19 HBT 119	+/+	+/+	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
7	20 HBT 123	-	+/+	-	+/+	+/+	+/+	+/+	+/-	+/+	+/+	+/+
8	21 HBT 71	No PCR	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
9	22 HBT 82	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
10	24 HBT 86	+/+	+/+	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
11	25 HBT 88	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
12	26 HBT 93	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
13	27 HBT 98	+/+	+/+	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
14	28 HBT 129	-	+/-	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-
15	29 HBT 134	-	+/+	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/-
16	30 HBT 107	+/+	+/+	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
17	32 HBT 136	-	+/+	-	+/+	+/+	+/+	+/+	+/+	+/-	+/+	+/+

TABLE V.1. Continued.

	DNA No	Promoter & A1	B1	C	D1	E1	F1	G	H1	H2	H3-1	H3-2
18	33 HBT 139	-	+/+	-	+/+	+/+	+/+	+/+	+/+	-	+/+	+/+
19	35 HBT 146	-	+/+	-	+/-	+/+	+/+	+/+	+/+	+/-	+/+	+/+
20	37 HBT 150	-	+/+	-	-	-	+/+	-	-	+/-	-	-
21	38 HBT 153	-	+/+	-	+/-	+/+	+/+	+/+	+/+	+/-	+/+	+/+
22	39 HBT 154	-	+/+	-	+/+	+/+	+/+	+/+	+/+	+/-	+/+	+/+
23	40 HBT 155	-	+/+	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
24	41 HBT 156	-	+/+	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
25	42 HBT 157	-	+/+	-	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+
26	43 HBT 158	-	+/+	-	+/+	+/-	+/-	+/+	+/+	+/+	+/+	+/+
27	44 HBT 159	-	+/+	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
28	45 HBT 160	-	+/+	-	+/+	+/+	+/-	+/+	+/+	+/+	+/+	+/+
29	46 HBT 161	-	+/+	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
30	47 HBT 162	-	+/+	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
31	48 HBT 163	-	+/+	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
32	49 HBT 164	No PCR	+/+	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
33	16 HBT 51	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
34	19 HBT 66	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
35	20 HBT 68	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
35	28 HBT 101	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
37	29 HBT 102	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
38	31 HBT 115	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+

2. Direct DNA Sequencing

Twenty-four exons from 23 individuals that caused an aberrant pattern in ddF reactions were sequenced to identify the changes. All, except two exons (exon D of HBT 93 and exon F of HBT 156), had sequence changes. HBT 93 had a sequence change in exon H (H2). Consequently, mutations were detected in 22 patients by ddF screening followed by DNA sequencing of the exon involving the change. All exons of HBT 156 and the rest of the hemophilia B patients who did not show any abnormal patterns by ddF were re-analyzed by direct DNA sequencing. In addition, nine new samples were also included in the direct sequencing analysis, bringing the total number of samples studied to 17. Eight patients were found to have sequence changes in various exons. The analyzed regions of FIX genes of these individuals by direct DNA sequencing are given in Table V.2. Finally, by ddF analysis and direct DNA sequencing 30 sequence changes were delineated in 47 suspected hemophilia B patients.

TABLE V.2. The segments of the FIX gene analyzed by direct DNA sequencing in 17 individuals. (+) and (-) signs show the analyzed and non-analyzed regions, respectively. Shaded boxes show the regions in which base changes were observed.

	DNA no	Promoter and A1	B1	C	D1	E1	F1	G	H1	H2	H3-1	H3-2
1	2 HBT 48	+	+	+	+	+	+	+	+	+	+	+
2	24 HBT 86	+	+	+	+	+	+	+	+	+	+	+
3	27 HBT 98	+	+	-	+	+	+	+	+	+	+	+
4	32 HBT 136	+	+	+	+	+	+	+	+	+	+	+
5	35 HBT 146	+	-	-	-	-	-	-	+	-	-	-
6	41 HBT 156	+	+	+	+	+	+	+	+	+	+	+
7	44 HBT 159	+	+	+	+	+	+	+	+	+	+	+
8	45 HBT 160	+	+	+	+	+	+	+	+	+	+	+
9	50 HTB 165	+	+	+	+	+	+	+	+	+	+	+
10	51 HBT 166	+	+	-	+	+	+	+	+	+	+	+
11	52 HTB 167	+	-	-	+	-	-	+	-	-	-	-
12	53 HBT 168	+	+	+	+	+	+	+	+	+	+	+
13	54 HBT 169	+	+	-	+	+	+	+	+	+	+	+
14	55 HBT 170	+	+	+	+	+	+	+	+	+	+	+
15	56 HBT 171	+	+	-	+	-	-	-	+	+	+	+
16	57 HBT 172	+	+	+	+	+	+	+	+	+	+	+
17	58 HBT 173	+	+	+	+	+	+	+	+	+	+	+

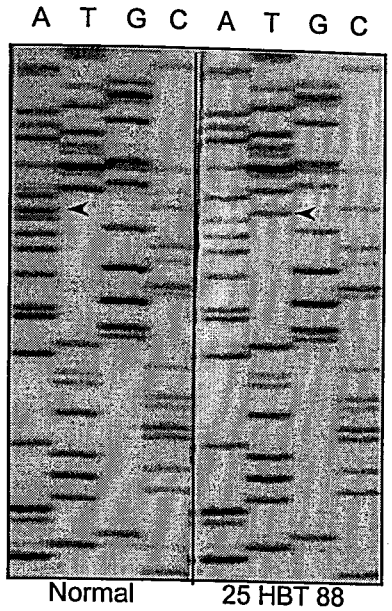
3. Novel Single Base Changes

Four of the identified 30 single base changes were reported for the first time in the framework of this thesis. These changes are described below. Others were already represented in the hemophilia B database.

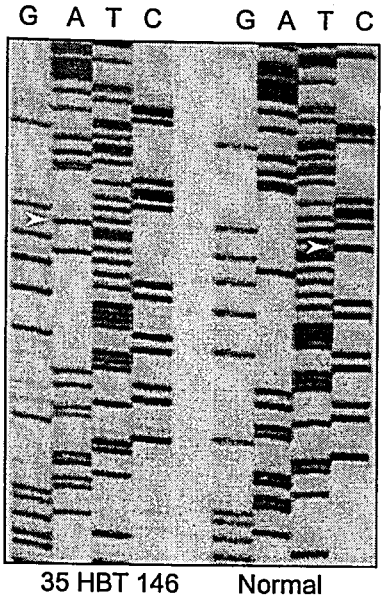
All exons, intron-exon boundaries and promoter region of the FIX gene of patient HBT 88 were screened with ddF, and an aberrant pattern was observed in the reaction performed with nested primer H3-2 of exon H. Sequencing analysis of this exon with the same primer identified a novel T to A transversion at nucleotide 31166 (Figure V.5.). This base change replaced Phe349 residue of the protein with an Ile. The change created a recognition site for restriction endonuclease *Mbo*I and abolished that of *Nsp*I, but was confirmed by re-sequencing of a newly prepared PCR since none of the enzymes were available. No other changes were identified in this patient. The clinical data for this patient is not available.

Similarly, all coding regions except for exon C, intron-exon boundaries and promoter region of the FIX gene of patient HBT 146 were screened with ddF. Abnormal patterns were not observed in any of the analyzed regions. The direct sequencing of this patient's FIX gene delineated a novel G to T transversion at nucleotide 30845 in exon H, creating a stop codon instead of a Glu at position 242 of the protein (Figure V.5.). The patient had a severe phenotype with a FIX:C of 1.3%. The mutation was also confirmed by re-sequencing starting from the PCR.

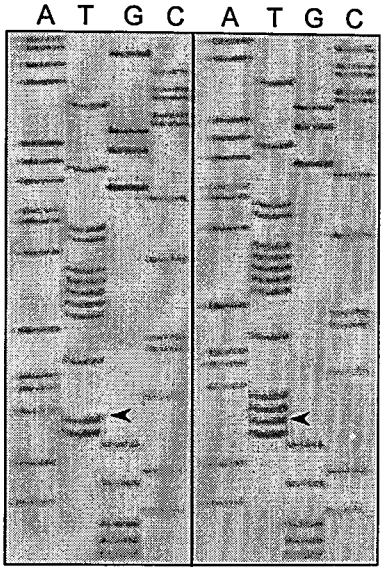
All exons, intron-exon boundaries and promoter region of the FIX gene of patient HBT 163 were screened by ddF. An aberrant pattern was observed in exon G ddF reaction. The subsequent sequencing analysis of this exon revealed the presence of a 2 bp (AA) insertion at position 30104 (Figure V.5.). This micro-



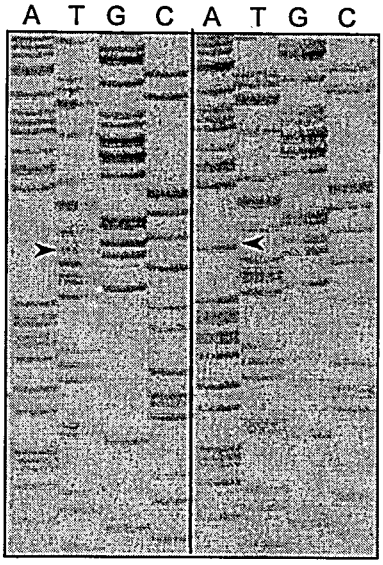
(a)



(b)



(c)



(d)

FIGURE V.5. Identification of novel single base changes in four patients. 8 per cent sequencing gels showing the sense strands of 25 HBT 88 and a normal individual **(a)**, the antisense strands of 35 HBT 146 and a normal individual **(b)**, the antisense strands of 48 HBT 163 **(c)**, and sense strands of 57 HBT 172 and a normal individual **(d)**.

insertion caused a shift in the frame of codons and resulted in conversion of Ile251 to a stop codon. The PTT of the patient is prolonged to 134" and he has a severe phenotype with a FIX:C of 1%. Inhibitors are not observed in the patient following replacement therapy.

A T to A transversion was detected at nucleotide 31171 after direct sequencing analysis of the likely functionally significant regions of patient HBT 172's FIX gene except for exon C (Figure V.5.). The base change resulted in the substitution of a stop codon at residue 350 with Cys. The patient has a severe phenotype with a 0.4% FIX:C.

4. Novel Gross Rearrangements

The two patients whose exon A did not amplify in ddF screening (Table V.1.) had gross rearrangements in the FIX gene, one of which was identified completely, and the nature of the other remained to be resolved.

a. Delineation of the End Points of a Large Deletion. The lack of amplification in exon A and any other aberrant pattern in the rest of the exons in the FIX gene of patient HBT suggested either a deletion spanning at least exon A or a large insertion interfering with the amplification of this region. The end points of a possible deletion were analyzed through a series of PCRs with primers designed from sequences in intron 1 and 5'UTR (Table III.3.). Initially, two polymorphic regions *BamH*I and *Dde*I (5' and 3' to exon A, respectively) were amplified. After positive results were obtained for these reactions, the region lying between nucleotides -341 and 5337 was scanned with various PCRs, using different sets of primers, none of which gave positive results (Figure V.6.). Approximately a 1200 bp fragment, instead of an expected 5980 bp one, was obtained from the PCR that was performed with primers –238 and 5719, and confirmed the presence of about

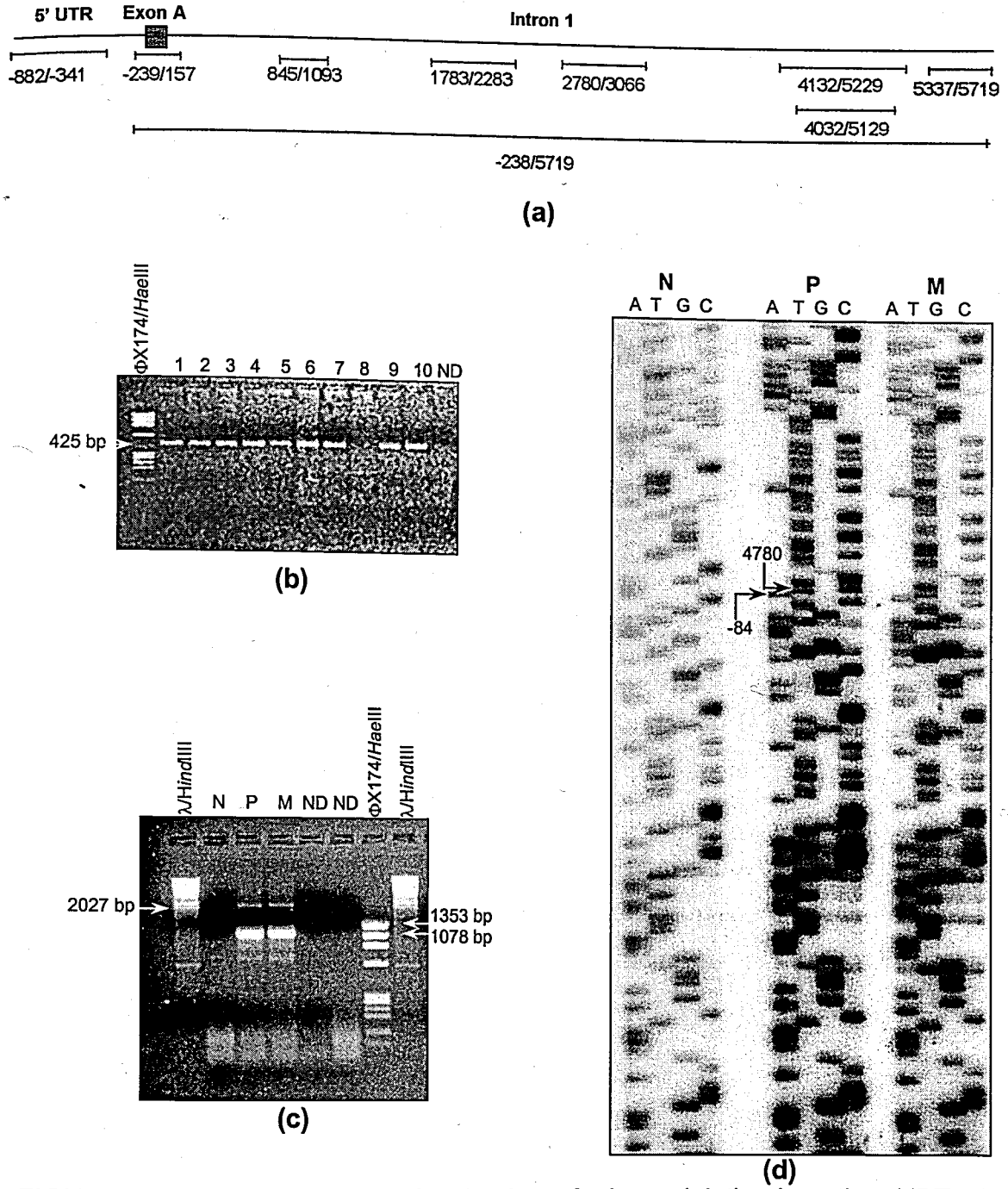


FIGURE V.6. The deliniation of end points of a large deletion in patient HBT 71. **A)** Schematic representation of the regions screened by a series of PCRs. Red and blue lines indicate amplified and non-amplified regions respectively. The green line shows the fragment that was sequenced for the identification of deletion junctions. **B)** 2 per cent agarose gel showing exon A PCRs. Lane 8 belongs to patient HBT 71. **C)** 1.5 per cent agarose gel showing the PCR results performed with primers –238/5719. **D)** 6 per cent sequencing gel showing the end points of the deletion. ND: No DNA, N: Normal; P: Patient; M: Mother of the patient.

a 4.8 kb-deletion. The sequencing of this fragment using nested primer F9-5'UTR(-239)17D delineated the end points of the deletion at nucleotides –84 and 4780. A normal control was used in each reaction. Since the expected size of the amplified fragment between bases –238 and 5719 is too large to amplify in a normal individual, the normal control FIX gene was amplified with exon A primers and sequenced with nested primer A1. The clinical data of the patient was not available. The mutation was confirmed by re-sequencing of a newly prepared PCR and mother of the patient was also found to carry the deletion.

b. An Unidentified Gross Rearrangement. Patient HBT 164 also did not amplify in exon A. The same approach to delineate the end points of a possible deletion was attempted. The initial step was the amplification of the two polymorphic regions, of which *Dde*I region amplified but B*amH*I failed, suggesting the involvement of a further upstream region. A series of PCRs were performed in intron 1 of the gene first to identify the 3' end of the possible abnormality. As a result of these reactions, it was identified that the FIX gene of the patient was normal at least beyond nucleotide 1780 (Figure V.7.). Then, the primer F9-(Sp6-23)-i1(2283)-40U was crossed with the available downstream primers in various PCRs as shown in Figure V.7. None of the reactions gave positive results. The next step should be the analysis of the patient DNA by Southern blot technique in order to understand the nature of the mutation.

All together, sequence changes were identified in 32 hemophilia B patients. Table V.3. summarizes all molecular and clinical data for these individuals. Out of these, nine patients (two from a previous study) had identical mutations in 4 different positions [123]. Mutations were observed almost in all exons, with the highest frequency in exon H.

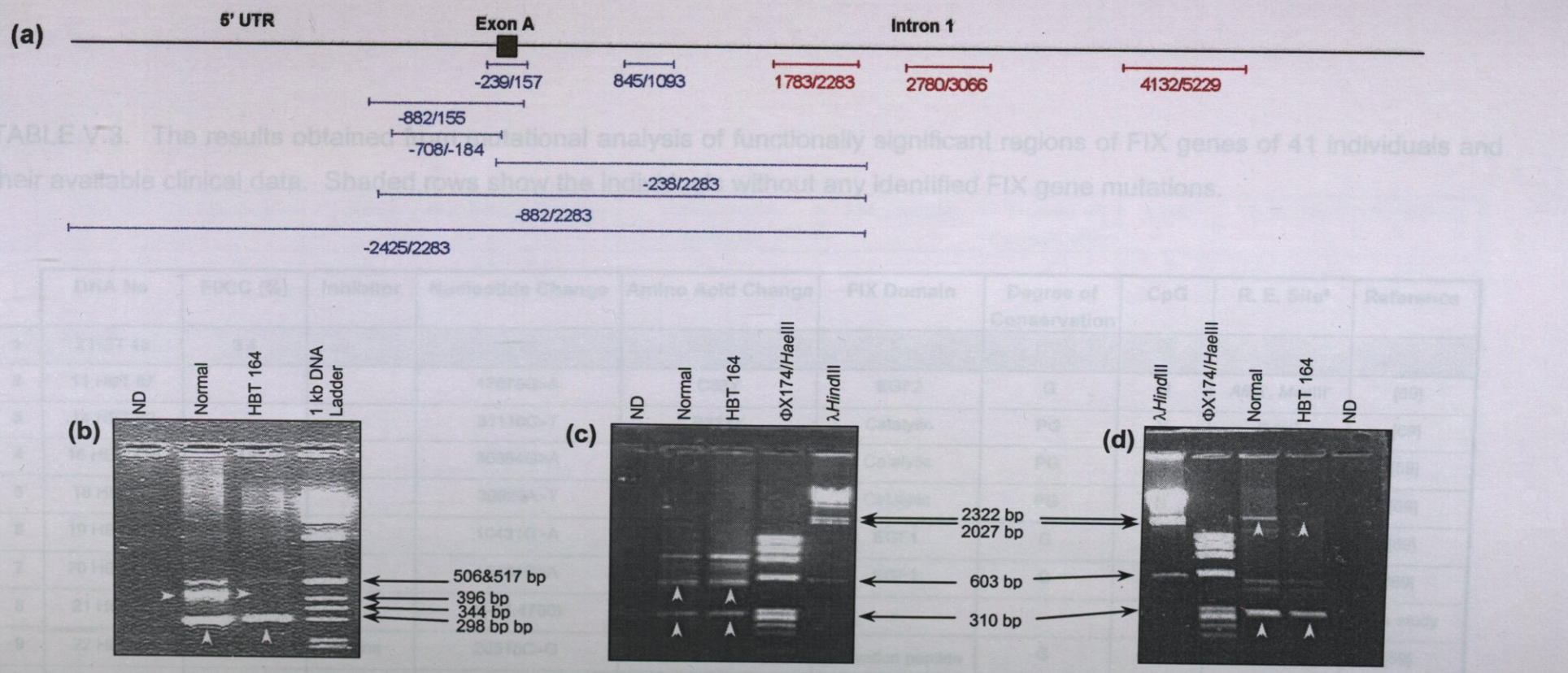


FIGURE V.7. Attempts for the identification of a gross rearrangement in patient HBT 164. **(a)** Schematic representation of the locations of the regions scanned with PCR. Red and blue lines indicate amplified and non-amplified regions, respectively. **(b)** 2 per cent agarose gel showing the lack of exon A amplification. **(c)** 2 per cent agarose gel showing the 1783/2283 amplification reaction. **(d)** 2 per cent agarose gel showing the lack of –238/2283 amplification. Arrowheads pointing the upper bands show the corresponding amplification results of each reaction. Arrowheads pointing the lower bands show the internal control PCR with *Taq*I primers.

TABLE V.3. The results obtained from mutational analysis of functionally significant regions of FIX genes of 41 individuals and their available clinical data. Shaded rows show the individuals without any identified FIX gene mutations.

	DNA No	FIX:C (%)	Inhibitor	Nucleotide Change	Amino Acid Change	FIX Domain	Degree of Conservation	CpG	R. E. Site[a]	Reference
1	2 HBT 48	3.4								
2	11 HBT 87			17678G>A	C88Y	EGF2	G	N	*AflIII*$^-$, *MaeIII*$^-$	[69]
3	14 HBT 62			31118C>T	R333X	Catalytic	PG	Y	*DdeI*$^+$	[69]
4	16 HBT 118	0.2		30864G>A	R248Q	Catalytic	PG	Y	-	[69]
5	18 HBT 56			30929A>T	I270F	Catalytic	PG	N	-	[69]
6	19 HBT 119	3		10431G>A	G60D	EGF1	G	N	*AciI*$^-$	[69]
7	20 HBT 123	0.1		10484G>A	E78K	EGF1	G	N	*MseI*$^+$, *DraI*$^+$	[69]
8	21 HBT 71			Δ(-84,4780)	-	-		-	-	This study
9	22 HBT 82	Moderate	None	20518C>G	R180G	Activation peptide	G	N	*AvaI*$^-$, *BsrI*$^+$	[69]
10	24 HBT 86	< 1								
11	25 HBT 88			31166T>A	F349I	Catalytic	PG	N	*MboI*$^+$, *NspI*$^-$	This study
12	26 HBT 93	2		31130C>T	L337F	Catalytic	PG	N	*BbsI*$^-$, *MboII*$^-$	[69]
13	27 HBT 98	2		6365G>A	R-4Q	Gla	PG	Y		[69]
14	28 HBT 129			30936T>G	L272R	Catalytic	PG	N	-	[69]
15	29 HBT 134			31133C>T	R338X	Catalytic	FS	Y	*TaqI*$^-$	[69]

TABLE V.3. continued.

	DNA No	FIX:C (%)	Inhibitor	Nucleotide Change	Amino Acid Change	FIX Domain	[a]Degree of Conservation	CpG	[b]R. E. Site	Reference
16	30 HBT 107	5		31202T>C	C361R	Catalytic	G	N	*NlaIII*⁻,*MaeIII*⁺	[69]
17	32 HBT 136									
18	33 HBT 139			31119G>A	R333G	Catalytic	PG	Y	-	[69]
19	35 HBT 146			30845G>T	E242X	Catalytic	PG	N	-	This study
20	37 HBT 150	1.3	Not tested	6365G>T	R-4L	Gla	PG	N	*HaeIII*⁻, *EaeI*⁻	[69]
21	38 HBT 153	1		20563C>A	Q195K	Catalytic	G	N	*Sty*⁻	[69]
22	39 HBT 154			20519G>A	R180Q	Activation peptide	G	Y	-	[69]
23	40 HBT 155	2.2		31218G>A	G366E	Catalytic	G	N	*MnlI*⁺	[69]
24	41 HBT 156	20.9								
25	42 HBT 157	0.5		31218G>A	G366E	Catalytic	G	N	*MnlI*⁺	[69]
26	43 HBT 158	7.8		30070G>A	C206Y	Catalytic	G	N	-	[69]
27	44 HBT 159									
28	45 HBT 160	30.3								
29	46 HBT 161	7.8	None	31170G>T	C350F	Catalytic	G	N	-	[69]
30	47 HBT 162	4	None	30864G>A	R248Q	Catalytic	PG	Y	-	[69]
31	48 HBT 163	1	None	30104InsAA	I251X	Catalytic		N	-	This study
32	49 HBT 164				-	-		-	-	This study
33	50 HTB 165	44.6								

TABLE V.3. continued.

	DNA No	FIX:C (%)	Inhibitor	Nucleotide Change	Amino Acid Change	FIX Domain	[a]Degree of Conservation	CpG	[b]R. E. Site	Reference
34	51 HBT 166	12.2	-	20518C>G	R180G	Activation peptide	G	N	*AvaI*$^{-}$, *BsrI*$^{+}$	[69]
35	52 HTB 167	3.9		112G>A	C-19Y	Signal pep.	FS	N	*RsaI*$^{-}$	[69]
36	53 HBT 168	Not determined								
37	54 HBT 169			30863C>T	R348X	Catalytic	PG	Y	-	[69]
38	55 HBT 170	3								
39	56 HBT 171	3.8		31130C>A	L337I	Catalytic	PG	N	*BbsI*$^{-}$, *MboII*$^{-}$	[69]
40	57 HBT 172	0.4		31171T>A	C350X	Catalytic	G	N	*AluI*$^{+}$	This study
41	58 HBT 173	1		30875C>T	R252X	Catalytic	NC	Y	*TaqI*$^{-}$	[69]

[a] G: Generic amino acid; PG: Partially generic amino acid; FS: FIX specific amino acid; NC: Non conserved amino acid.

[b](+) sign indicates that the mutation creates a recognition site for the given enzyme, whereas (-) sign indicates that the mutation abolishes an already existing one.

B. Haplotype Analysis of the Factor IX Gene

Haplotypes of 41 patients who were analyzed for FIX mutations were constructed using six polymorphic regions, *BamH*I, *Dde*I, *Xmn*I, *Taq*I, *Mnl*I, and *Hha*I (Table V.4.). These haplotypes possibly indicate whether the recurrent mutations occur independently or not, or they can be used to trace the mutant allele in carrier detection or prenatal diagnosis performed by linkage analysis.

TABLE V.4. The haplotypes of the 41 patients. Numbers given next to each haplotype show the group number.

	DNA#	*BamH*I	*Dde*I	*Xmn*I	*Taq*I	*Mnl*I	*Hha*I
1	2HBT48	-	380	-	-	-	-
2	11HBT87	-	380	+	+	+	-
3	14HBT62	-	430	-	-	-	-
4	16HBT118	-	430	-	-	-	+
3	18HBT56	-	430	-	-	-	-
4	19HBT119	-	430	-	-	-	+
4	20HBT123	-	430	-	-	-	+
5	21HBT71	-	380	-	+	-	-
6	22HBT82	-	380	-	-	-	+
2	24HBT86	-	380	+	+	+	-
1	25HBT88	-	380	-	-	-	-
3	26HBT93	-	430	-	-	-	-
7	27HBT98	-	380	+	+	+	+
7	28HBT129	-	380	+	+	+	+
8	29HBT134	-	430	-	+	-	-
9	30HBT107	+	380	-	-	-	+
4	32HBT136	-	430	-	-	-	+
1	33HBT139	-	380	-	-	-	-
10	35HBT146	-	380	-	+	-	+
2	37HBT150	-	380	+	+	+	-
11	38HBT153	-	380	+	-	+	+
	39HBT154		430	-		-	-
3	40HBT155	-	430	-	-	-	-
7	41HBT156	-	380	+	+	+	+
10	42HBT157	-	380	-	+	-	+
7	43HBT158	-	380	+	+	+	+
4	44HBT159	-	430	-	-	-	+
5	45HBT160	-	380	-	+	-	-
8	46HBT161	-	430	-	+	-	-
12	47HBT162	-	380	+	-	+	-
11	48HBT163	-	380	+	-	+	+
1	49HBT164	noPCR	380	-	-	-	-
6	50HBT165	-	380	-	-	-	+
3	51HBT166	-	430	-	-	-	-
6	52HBT167	-	380	-	-	-	+
7	53HBT168	-	380	+	+	+	+
1	54HBT169	-	380	-	-	-	-
7	55HBT170	-	380	+	+	+	+
4	56HBT171	-	430	-	-	-	+
4	57HBT172	-	430	-	-	-	+
13	58HBT173	-	380	-	+	+	-

Thirteen different haplotypes were observed in this sample of patients. Analysis of HBT 154 was not complete since the DNA sample was not sufficient; therefore, a group number was not assigned to this patient. The most common haplotype was group number 4 (-, 430, -, -, -, +) with a frequency of 17.5 per cent (Figure V.8.).

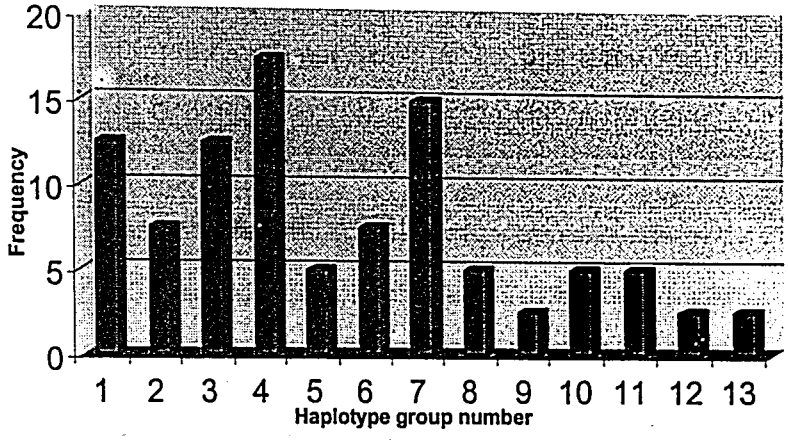


FIGURE V.8. The frequencies of the haplotypes observed in this study. Haplotype group numbers are as indicated in Table V.4.

C. Analysis of Intron 1cRY(i) Region

The hypervariable region located in intron 1 of the FIX gene, if analyzed by direct sequencing, show differences in genotypic variation among individuals and populations. A total of 85 alleles were analyzed for this region by direct sequencing. Fourty-one of these individuals were randomly chosen from different geographic regions of the country. The rest were the Turkish hemophilia B patients analyzed in this study and elsewhere [123, 124]. A novel allele was observed in one individual. Figure V.9. shows the agarose and sequencing gel patterns of different alleles. Figure V.10. shows the base composition of alleles observed in the Turkish population and Table V.5. shows the frequencies of these alleles.

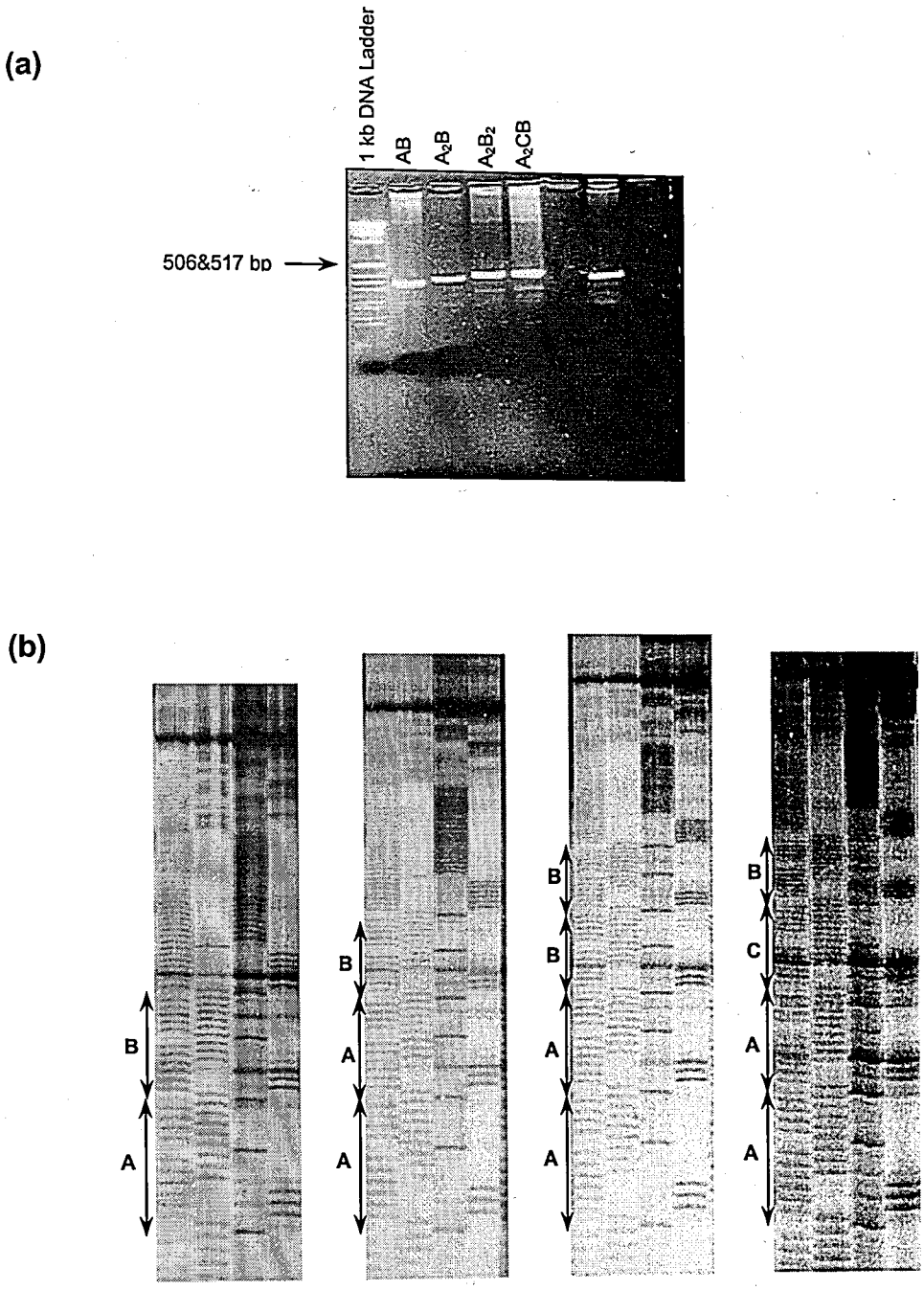


FIGURE V.9. The four different alleles of intron 1 cRY(i) identified in Turkish population. **a)** 2 per cent agarose gel showing the size differences of the alleles. **b)** 8 per cent sequencing gel showing the sequences of these alleles.

Allele AB [(GT)(AC)$_3$(AT)$_3$(GT)(AT)$_4$]$_1$ [(GT)(AC)$_3$(AT)$_3$(GT)(AT)$_5$]$_1$

Allele A_2B [(GT)(AC)$_3$(AT)$_3$(GT)(AT)$_4$]$_2$ [(GT)(AC)$_3$(AT)$_3$(GT)(AT)$_5$]$_1$

Allele A_2B_2 [(GT)(AC)$_3$(AT)$_3$(GT)(AT)$_4$]$_2$ [(GT)(AC)$_3$(AT)$_3$(GT)(AT)$_5$]$_2$

Allele A_2CB [(GT)(AC)$_3$(AT)$_3$(GT)(AT)$_4$]$_2$ [(GT)(AC)$_3$(AT)$_3$(GT)(AT)$_6$]$_1$
[(GT)(AC)$_3$(AT)$_3$(GT)(AT)$_5$]$_1$

FIGURE V.10. The base compositions of the repeat units of intron 1 cRY(i) alleles identified in the Turkish population. Allele A_2CB was identified for the first time in this study.

TABLE V.5. The frequencies of the intron 1 cRY(i) alleles observed in 85 Turkish individuals.

Allele	Number Identified	Frequency (%)
AB	57	67.0
A_2B	5	5.9
A_2B_2	22	25.9
A_2CB (New)	1	1.2

D. Carrier Detection and Prenatal Diagnosis

Carrier determinations and prenatal diagnosis of hemophilia B can be conducted accurately for Turkish hemophilia B families, based on the DNA sequencing and haplotype analysis methods established in our laboratory. Carrier detections were performed for 20 individuals from 11 families at risk. The carrier status of 13 individuals from 5 families, in which mutations were identified, were performed by direct sequencing, others by linkage analysis. Three pregnant females at risk applied for prenatal diagnosis. Two of the fetuses were detected as healthy but one of them was aborted spontaneously. The third individual had a spontaneous abortion before the chorionic villus sampling. Carrier detection studies performed for this family is given in Figure V.11. as an example.

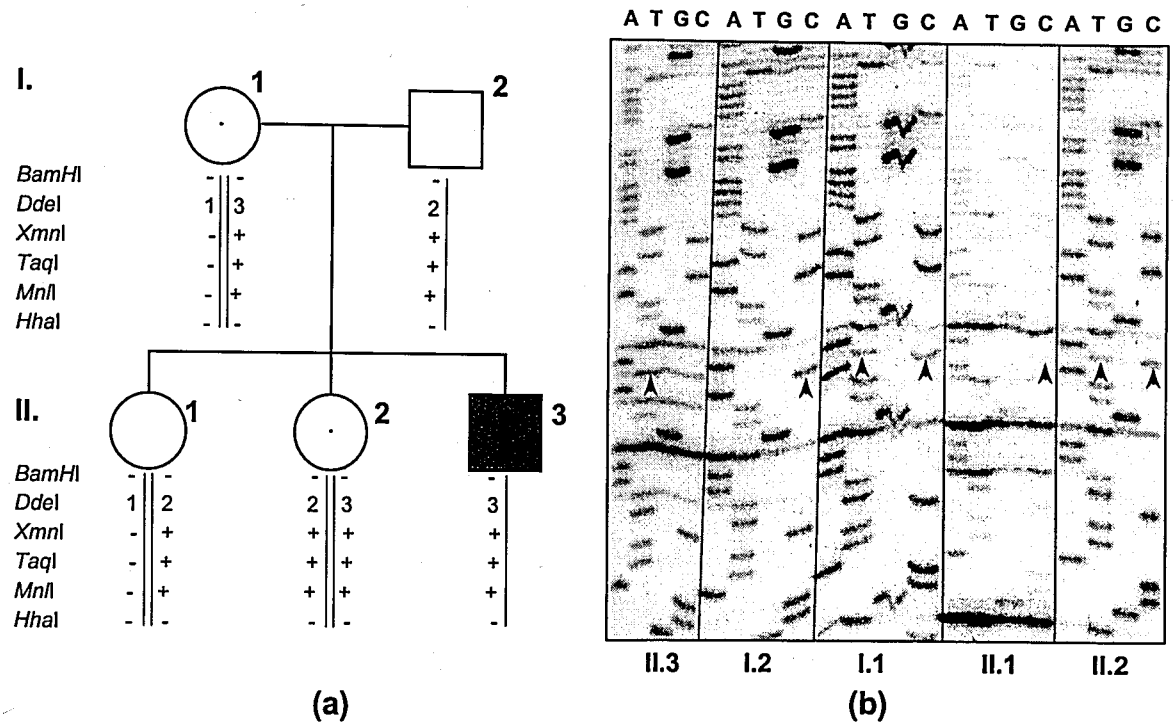


FIGURE V.11. Linkage and sequencing analyses performed for carrier diagnosis in a hemophilia B family. **a)** The pedigree of the family showing the linkage analysis, in which the mutant allele is traced by the haplotype of the patient. According to linkage analysis, individual II.2. is a carrier, while individual II.1. is not. **b)** 6 per cent sequencing gel showing the carrier detection study performed by direct sequencing analysis.

VI. DISCUSSION

A. Mutations Identified in Turkish Hemophilia B Patients and Genotype/Phenotype Correlations

Causative mutations have been delineated in 32 Turkish hemophilia B patients, including four novel point mutations and a large deletion. Four of these mutations occurred in more than one Turkish hemophilia B patient, and 16 mutations have occurred at hot-spot CpG sites. Two of the mutations (30936T>G and 31202T>C) created new CpG sites on exon H of the gene. Figure V.1 shows the distribution of these mutations on the functional domains of the FIX gene.

Identification of a novel change in a gene gives an insight to the understanding of the function of the protein when genotype and phenotype correlations are formed. For this purpose, the possible effects of novel mutations on the protein function is discussed below.

The 4.8 kb deletion identified in patient HBT 71 is the mutation causing hemophilia B in this patient. The coding regions, and splice junctions were analyzed by ddF and no other changes were found. The deletion effects the transcriptional control of the gene by removing the binding sites of trans-acting transcriptional factors NF-1L (-99/-77), ARE (-34/-23), HNF4 (-27/-19), an unidentified factor (-15/-1), and C/EBP (+4/+19). Moreover, the complete sequence of exon A and a large portion of intron 1 are also deleted. This patient is most probably a CRM^- patient since the deletion presumably blocks the mRNA synthesis. Even if the mRNA synthesis is not blocked completely, the defected factor IX will not be post-translationally modified and secreted since exon A codes for the signal peptide that leads the protein to the endoplasmic reticulum for these processes. Moreover, intron 1 is also reported to have slight effects on the

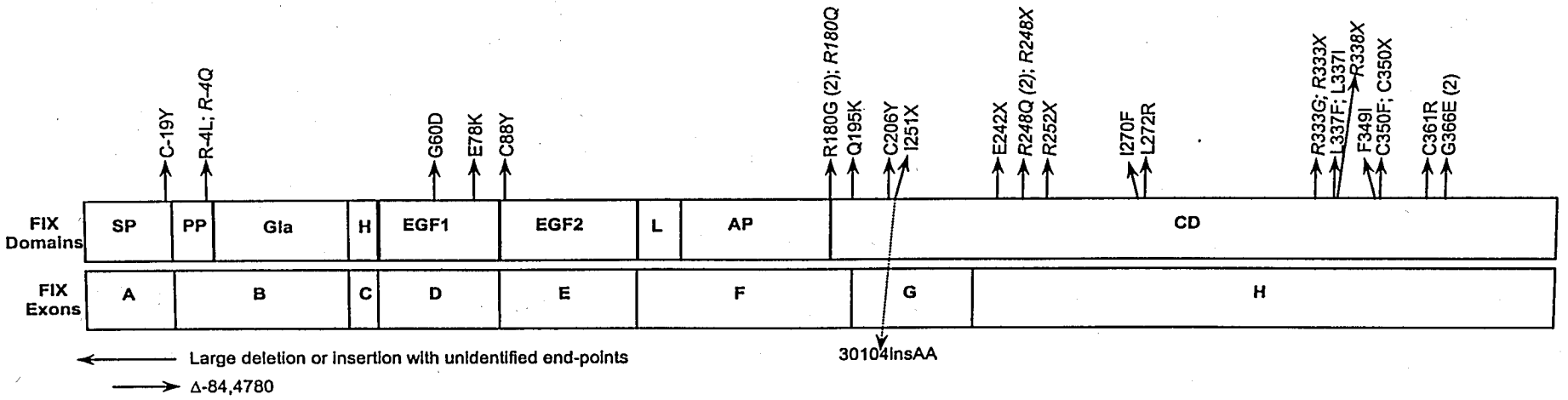


FIGURE VI.1. The distribution of point mutations, identified in this study, on the domains of coagulation FIX. The lower part of the figure shows the exons of the gene, and the locations of one large deletion as well as the putative location of the presumed large deletion or insertion. The upper part of the figure shows the FIX domains corresponding to the exons of the genes, and the locations of the point mutations as well as the stop codon created by the shifted frame because of two base pair insertion (the corresponding location on the gene is indicated with the dashed arrow). The numbers in the parantheses show the occurrence number of the mutation, and the mutations that are given in italic form show the CpG transitions. SP: Signal peptide; PP: Propeptide; H: Hydrophobic domain; L: Linker peptide; AP: Activation peptide; CD: Catalytic domain.

expression of the gene. Unfortunately, no clinical data is available for this patient such as the FIX:Ag, FIX:C and inhibitor development.

Although it is not correct to score a mutation unless it is completely identified by sequencing, we scored the gross change in patient HBT 164 as a novel mutation, based on the low frequency of deletions observed in the FIX gene and the lack of a hot-spot for such rearrangements.

The 31166 T to A transversion identified in patient HBT 88 is the causative mutation for a number of reasons. First, no other changes were found in the patient after screening all of the likely functionally significant regions of the FIX gene by ddF. Second, the mutation replaces the normal Phe349 residue with an Ile. Both amino acids are non-polar, but Phe, which has an aromatic side chain composed of a phenyl group, differs in size and shape from Ile that has an aliphatic hydrocarbon side chain. Moreover, this residue is placed in a disulfide loop formed by Cys336 and Cys350, and the partially generic conservation of this amino acid may indicate its functional importance but not the structural one. No clinical data is available for the patient but he is expected to have a moderate or mild CRM^+ phenotype because of the nature of his mutation.

The patient HBT 146 has only a G to T transversion at position 30845 of his FIX gene. No other changes were identified in the analyzed regions (except exon C) of the FIX gene by ddF. This is expected to be the causative mutation since it creates a prematurely terminated protein at amino acid residue 242, which is partially generic. The secretion of this truncated protein might slightly be reduced, because a number of C-terminal residues of the protein (403-415) were shown to be essential for its secretion [125]. More importantly, it is functionally defective since it does not contain the residues forming cofactor (FVIII) binding sites (301-303 and 333-339), substrate binding (FX) pocket (359,386, and 396), and two important amino acids (269 and 365) of the catalytic triad, which is responsible for

FX activation. The clinical data for the patient is not available, but he is expected to have a CRM^{red} phenotype.

The 12 year-old patient HBT 163 has a two base pair insertion at position 30104 of his FIX gene. This micro-insertion is the cause of hemophilia B in this patient, because it causes a shift in the codon frame and results in the formation of a stop codon at residue 251 of the protein. Similar to the reasons stated for patient HBT 146, this patient is also suspected to have a CRM^{red} phenotype. The molecular findings of the patient correlate well with his severe phenotype with a 1% FIX:C. His FIX:Ag level is not studied, but he has not developed inhibitors after replacement therapy. The PTT of the patient is prolonged to 134" whereas normal individuals have a value between 35"-45". Exon A and exon C are not analyzed for this patient, therefore, he might have other changes in these exons. However, even if there is another change, this frameshift mutation seems to be sufficient to cause the disease.

The patient HBT 172 has a T to A transversion at nucleotide 31171. This causative mutation produces a truncated protein at residue 350, which is a Cys in normal FIX. No other changes were observed in the patient after sequencing all exons except exon C. The patient has a severe phenotype with a 0.4% FIX:C. He is 7 years old and diagnosed as hemophilia B because of having prolonged bleeding and after developing hematomas at injection points. He also developed hemarthroses twice. The severe phenotype agrees well with his mutation, which disrupts the catalytic function of the protein by removing the substrate binding pocket and the active site residue Ser 365. The secretion of the protein might slightly be decreased as stated for patient HBT 146, but this is not confirmed since no information is available about his FIX:Ag level.

B. The Profile of FIX Mutations

The mutation profiles are helpful to understand whether the mutations occur due to environmental factors or endogenous processes. If environmental factors as mutagens, life-style, dietary habits, and geographic variations are the primary cause of the disorder, the pattern of mutations is expected to be specific to each population. However, if endogenous processes are responsible, the pattern observed worldwide should be more or less similar. Hemophilia B mutations constitute a good model to test this hypothesis, since until the use of replacement therapy, the reproductive fitness of the patients was low and the occurrence of the disease in a family is mostly sporadic. The population-based studies for hemophilia B, including European, Asian, Caucasian, and different ethnic groups of American populations revealed the mutation profiles of these populations, and the comparison of the data obtained all over the world, showed that the mutation profiles of the studied populations are more or less similar except for the Chinese population. This population is assumed to have a different mutation profile in one study [126]. The comparison of the mutation profile that is constructed by the analysis of 76 Chinese patients with the mutation profile constructed by a similar number of Caucasian mutations revealed the presence of 18 versus 36 per cent CpG transitions, 11 versus 34 per cent non CpG transitions, and 0 versus 11 per cent large deletions, suggesting that this population may be exposed to different mutational mechanisms.

The data obtained in this study together with the data from three previous studies [123, 124, 127] revealed the mutation profile of the FIX gene for the Turkish population, which is similar to the one observed in other studies. Table VI.1. and Figure VI.2. show the mutation profile of the Turkish population and its comparison with the profile revealed by the FIX mutation database, respectively. This comparison showed that they are not much different in the frequency of missense and nonsense mutations. However, in the Turkish population, in frame deletions, cryptic splice-site mutations and promoter mutations are not observed.

This is most probably not an indication of an environmental or genetic difference, but because of the large difference in the sample sizes (45 versus 689 independent events). This difference should not be compared to the difference observed in the Chinese population (if there is any), because in that study similar sized samples were compared from two populations. However, our data was compared with that of the database, which includes data from all populations.

TABLE VI.1. The classification of the mutations identified in the Turkish population.

Mutation Type	This study		This study+previous studies	
	# Identified	% Identified	# Identified	% Identified
♦ Missense	23	71.9	31	69
- Transition	*14*	*43.8*	*20*	*44.5*
- Transversion	*9*	*28.1*	*11*	*24.5*
♦ Nonsense	6	18.8	8	17.8
- Transition	*4*	*12.5*	*6*	*13.3*
- Transversion	*2*	*6.3*	*2*	*4.5*
♦ Frameshift	1	3.1	2	4.5
♦ Large deletions and insertions	2	6.3	3	6.7
♦ Splice junction	–	–	1	2.2
TOTAL	32	100.0	45	100.0

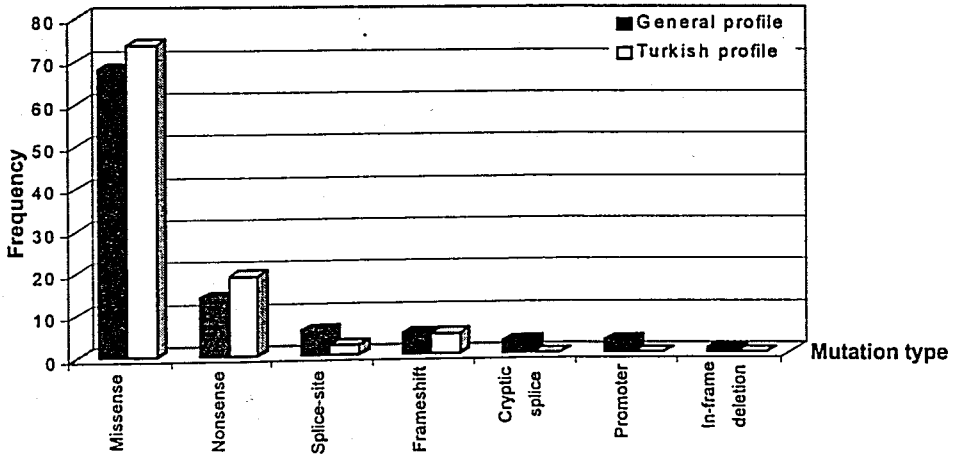


FIGURE VI.2. The comparison of the Turkish mutation profile with the hemophilia B database.

According to the general pattern, the transitions at CpG sites account for more than 25 per cent of the FIX mutations. Transitions occur more frequently than transversions at CpG dinucleotides, however, their occurrence rates are closer at non-CpG sites. Similar to the general pattern, the frequency of transitions at CpG dinucleotides is 32.5 per cent in the Turkish population, and in overall, transitions and transversions occur 40 per cent at these mutational hot-spots (Table VI.2.). Moreover, the frequencies of transitions and transversions at non-CpG sites are 35 and 25 per cent, respectively, which is also similar to the general pattern.

TABLE VI.2. The distribution of transitions and transversions between CpG versus non-CpG sites in Turkish hemophilia B patients.

	# at CpG sites	% at CpG sites	# at non-CpG sites	% at non-CpG sites	Total (%)
Transitions	13	*32.5*	14	*35*	*67.5*
Transversions	3	*7.5*	10	*25*	*32.5*
Total	16	*40*	24	*60*	*100*

The similarity between mutation profiles and the frequency of transitions at CpG dinucleotides among populations confirm that endogenous processes are responsible for FIX mutations, rather than environmental factors. This data obtained from the Turkish population is also contradictory to the occurrence of different frequencies of CpG versus non CpG transitions in the Chinese population.

C. The Mechanism of Mutations

One of the best explained endogenous processes causing a genetic disease is the spontaneous deamination of a 5mC residue to a T at CpG dinucleotides. CpG dinucleotides are hot-spots for transversions as well as

transitions. A proposed mechanism for such mutations depends on the knowledge of correction of G:T mismatches created by delamination of 5mC. The T residue is said to be removed by a DNA glycosylase, and the gap is filled by a repair mechanism which was suggested to be error-prone because of the possible insertion of a base that results in a transversion [128]. A slipped mis-pairing model is proposed for mutations at non-CpG sites, which is thought to be mediated by repetitive DNA sequences around the mutated base(s) [129]. Other chemical processes such as the presence of highly reactive free radicals and toxic materials, and enzymatic processes like depurination are also known to cause single base substitutions. Small insertion and deletions are mainly explained by slipped-strand mechanism which depends on the formation of a loop either in template DNA (deletion) or in newly synthesized DNA (insertion). Repetitive, palindromic and directly repeated DNA sequences in the vicinity are believed to mediate deletions and insertions (small or large). Moreover, homologous or nonhomologous recombination is a more probable mechanism for large deletions and also for large insertions. We did not observe any unusual mechanism in our patients with novel mutations.

D. Haplotype Analysis

Recurrent mutations were observed at four positions in the Turkish population. The 31218G>A transition was observed in two patients analyzed in this study. The completely different haplotypes of these patients (-, A_2B_2, -, -, -, - and -, AB, -, +, -, +) showed that the mutations occurred independently. Similarly, the 30863C>T transition was observed in two unrelated patients, confirmed by their different haplotypes (-,AB, -, -, -, - and -, AB, +, +, +, +). The first patient was included in this study and the other in a previous study of Turkish hemophilia B patients [123]. The other recurrent mutation was the 30864G>A transition, which was observed in two cases of this study and in one patient from a previous study [123]. The haplotypes of both patients from this study differed from each other

(-,A_2B_2, -, -, -, + and -, AB, +, -, +, -), and from the other patient's haplotype (-, AB, +, +, +, +) suggesting the mutations are specific to each family. This transition was suggested as a new potential founder effect in the U. S. Caucasian population in a study including 276 samples of this population [130]. This mutation was observed in 4.4 per cent of the sample and out of these 58 per cent had the same haplotype which differed from the haplotypes observed in our two patients. The remaining 42 per cent shared four different haplotypes, one of which (-,A_2B_2, -, -, +) is similar with that of our first patient. Although *Mnl*I site was not included in their haplotype analysis, the linkage disequilibrium observed between *Xmn*I, *Taq*I and *Mnl*I sites indicate that most probably they have the same haplotype. Haplotype analysis is not the only criteria to prove the independent origin of mutations, they may also be *de novo* recurrent mutations. Independent origin of the mutations can ultimately be explained by finding the origin of the mutations in those families. The last recurrent mutation found in this study is the 20518C>G transversion. Again, both patients with the same mutation have different haplotypes (-, AB, -, -, -, + and -, A_2CB, -, -, -, -). The last three of the recurrent mutations are observed at Arg residues which are hot-spots of mutations since all CGN codons code for this residue. The hot-spot nature of the mutant site further explains the *de novo* occurrence of these recurrent mutations.

In one study, eight different ethnic groups were studied for five polymorphic loci other than *Dde*I. Haplotypes were classified into three classes according to their frequency, heterogeneity and ethnic origin [88]. Class I haplotypes are common to all ethnic groups, and characterized by a (-) allele for the four except *Hha*I (original haplotypes). *Hha*I site showed a strong ethnic varience, with a 87 per cent frequency of (+) allele in Caucasians and Africans, and with a 64 per cent frequency of (-) allele in Orientals. They observed one exception to this class where *Taq*I allele was (+) (recombinant haplotype). Class II haplotypes were (-) for *BamH*I and (+) for *Mnl*I sites. This class, including seven subtypes, was predominantly European and accounts for 14 percent of all haplotypes. Class III haplotypes, which account for nine per cent of all, are characterized by a (+) allele for *BamH*I and specific to African populations. This study shows that, X

chromosome with the half-rate of recombination compared to autosomes, presents valuable information for molecular evolution studies in addition to Y chromosome and mitochondrial DNA.

In a previous analysis of the Turkish population with 37 normal DNA samples, the common haplotype was found in 65 per cent of the population, and of this, *Hha*I (-) allele was represented with 48 per cent [90]. The rest of the haplotypes were of class II type. In this study, class I haplotypes also have a 65 per cent frequency, and the HhaI (-) allele constitutes 45 per cent of this. However, the frequency of class II alleles drops to 32.5 per cent compared to the other study on the Turkish population. This decrease is observed because of the presence of a class III haplotype with a frequency of 2.5 per cent.

Although the sample size is small for a population based study, both studies are in accordance with each other, and with the study mentioned above. This shows us that there is no bias in the analysis of normal or patient DNAs by means of polymorphic regions and haplotype analysis for the FIX gene. This is also an expected result since there is no association of certain haplotypes to certain types of mutations as observed in some diseases such as cystic fibrosis. This is most probably due to the extremely heterogeneous and sporadic nature of mutations observed in the FIX gene.

E. Turkish Mutation Database

Our mutational analysis of Turkish hemophilia B patients is complete with a detailed information about their haplotypes and clinical information that helps to establish relationships between haplotype, genotype, and phenotype. This information is compiled in a national database that is available on internet [131].

We feel that the establishment of such national databases providing detailed information about the patients's phenotype and genotype is necessary for further computational analysis and comparisons between different ethnic/national groups.

F. Analysis of the Intron 1 cRY(i) Region

Alternating purine-pyrimidine repeats are an abundant source of polymorphism. They are found extensively in eukaryotic DNA. Cryptic RY(i) are also purine-pyrimidine repeats in which none of the RY predominates.

The structure of such a cryptic repeat region in intron 1 of the FIX gene was identified as shown by the hypervariable nature of two repeat units called A and B. This region show high genotypic variation among individuals and populations as found in a study of more that 1700 alleles [84]. Two types of alleles were identified which were derived from the primordial allele AB through recombination (A_nB) or polymerase slippage (A_nB_2) which showed ethnic and race specificity.

The cryptic RY(i) repeat in intron 1 was initially studied by PCR (named as *Dde*I) to construct haplotypes for the Turkish patients. The same region was also analyzed by sequencing to see further differences in individual haplotypes of patients with recurrent mutations. Normal samples were also included in this analysis to see the distribution and variability of the alleles in the Turkish population. A novel sequence, named A_2CB, was found in this study. In this allele, C is B with an additional (AT) at 3' end of the repeat. Such hypervariable regions are naturally prone to mutations. This AT dinucleotide insertion could be a *de novo* mutation in this patient. If it is a stable new allele, it would be observed in a larger sample size of the population. Therefore, it would be interesting to collect

a large number of samples from different ethnic groups living in this country to see the presence of A_2CB or other alleles and the extent of genetic variability within the Turkish population.

The new allele, A_2CB, was observed in a patient DNA sample (HBT 166). It could indicate a mutation causing the disease. However, it is not the case and there are a number of reasons for this. First, the causative mutation in this patient was identified. Second, another patient who has the same mutation had an AB allele. Last, in the study mentioned above, the effect of this mutation prone hypervariable region was analysed and normal individuals, who do not have any bleeding problems,having AB and A_2B_2 alleles were found to have similar levels of FIX:C. These individuals were also from the same age group, since with increasing age FIX:C levels increase.

This hypervariable region also has evolutionary importance. Chimpanzees and orangutans were analyzed for this region and it was found that chimpanzees have a long stretch of cRY(i) but not a repeating unit structure. In contrast, orangutans have a similar A/B type pattern where the structures of A and B resemble the A/B units observed in humans [84].

G. Patients with Unidentified Mutations

We could not find any sequence changes in nine patient samples. The reason for this could be clinical misdiagnosis of patients with another bleeding disorder, such as hemophilia A instead of hemophilia B. We had such cases who had been sent with hemophilia A diagnosis, but later turned out to be hemophilia B patients rather than A, and vice versa. Therefore, standardization of these tests is required for reliable diagnosis. The clinical tests for these individuals have to be

repeated to confirm if they are really hemophilia B patients, or not. If their hemophilia B diagnosis is confirmed, then, the causative mutations can be suspected to reside in other regions such as in ntrons that were not analyzed. All likely functionally regions of these patients were analyzed by direct sequencing in addition to initial ddF screening. They may have deep intronic mutations, or mutations in yet unknown regulatory regions. We normally analyze the splice junctions but in one of the regions (exon D), only 15 bases following splice site could be read, but it is unlikely for all of these individuals to have the causative mutations in this region. Moreover, deep intronic mutations are very rare even below one per cent.

H. Dideoxy Fingerprinting

Dideoxyfingerprinting is a hybrid of SSCP and Sanger's dideoxy chain termination sequencing method. When compared to 70-80 per cent mutation detection efficiency of SSCP, ddF is more efficient with a capability of detecting approximately 100 per cent of mutations [95].

In our study, we used ddF to screen 30 patients to detect their FIX gene mutations. We initially found mutations in 22 of the patients, making the efficiency of the method (73 per cent) same as usual SSCP. However, we could not observe any mutations by direct sequencing analysis in six of the eight patients, who did not show any abnormal pattern by ddF. Thus, the efficiency of the method was 94 per cent, detecting 22 mutations in 24 samples. The two mutations that could not be identified by ddF are 6365G>A and 30845G>T. The possible explanations are mentioned briefly in the succeeding paragraphs.

The efficiency in detection of mutations by ddF is strongly related both to the quality of experimental conditions and to the interpretation of data. Any small change in the quality of the reaction, the gel and the electrophoresis conditons alter the results dramatically. For example, if one of the plates was not covered with silicon reagent, the gel might stretch easily while disassembling the plates after electrophoresis, so the bands residing in that area become impossible to interpret. Temperature changes are also important, for high temperature changes may alter the secondary structure of the DNA fragments. It has been suggested to run the gels at 8°C rather than at room temperature. Moreover, the temperature of the gel is not uniform during electrophoresis when a big sequencing apparatus is used, being more at the upper parts of the gel. This may be the reason of failure in detecting the 30845G>T mutation, because we performed electrophoresis at room temperature and this base is near the 3' of the sequenced fragment and resides in the upper parts of the gel. To eliminate this obstacle, an apparatus with a cooling system should be used. Such machines both cool the gel and keep the temperature stable during electrophoresis. Placing a fan facing the gel (not performed in our study) might be useful for distribution of the temperature uniformly in the gel.

Gel matrix is another important factor in the efficiency of ddF. Several gel matrices were tried in one study and regular polyacrylamide gels were found to be the poorest quality gels in detecting altered ddF fragments. Special gels like GeneAmp™ and MDE™ gels are of similar quality and are high capacity gel matrices [132]. We mostly used MDE gels of in this study.

The reaction quality also is very important. Some reactions look very nice at the 5' end but fade out through the 3' end. These reactions may show altered fragments, but should never be scored as reliable reactions and should be repeated. Because, the observed altered fragments are caused by the poor termination reactions, producing multiple nonspecific fragments by the enzyme used (AMV reverse transcriptase in this study).

The type of mutation, the base composition of the analyzed genomic region, and the position of the mutation relative to the 3' end are the other important factors affecting the efficiency of ddF.

VII. CONCLUSION

This study included the mutational analysis of 41 individuals with hemophilia B either by dideoxy fingerprinting or direct DNA sequencing and revealed the mutations of 32 individuals, including four novel single base changes and two novel gross rearrangements. A mutation profile was constructed for the Turkish population, and it was found to be similar to the general profile observed in the world, showing that endogenous processes are responsible for the mutations affecting the factor IX gene. The novel mutations identified in this study are expected to contribute to the knowledge of genotype-phenotype correlations in hemophilia B, thus improve the gene therapy studies.

Haplotypes were constructed for all patients utilizing the six polymorphic regions of the FIX gene. Thirteen different haplotypes were observed. Recurrent mutations were seen at 4 positions and it was shown that these mutations occurred independently in each case, by the presence of different haplotypes in each patient.

All patient data was compiled in a national database, which efficiently shows the mutation-phenotype-haplotype relations.

The analysis of the hypervariable region in intron 1 of the FIX gene by direct sequencing in 85 Turkish individuals revealed the presence of Caucasian population specific alleles in Turkish population, as well as one novel allele identified in this study.

Carrier detections were performed for 20 individuals from 11 families and prenatal diagnoses were given to two pregnant females at risk.

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