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A new polymorphism in the factor VIII gene for prenatal diagnosis of hemophilia A

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ABSTRACT

A restriction fragment length polymorphism (RFLP) has been found in the gene for clotting factor VIII. Defects in this gene are the cause of hemophilia A. The DNA polymorphism affects an XbaI site in intron 22 of the gene. Two alleles occur in a frequency of 59 and 41 percent of the X chromosomes tested. Furthermore, about 25 percent of females who are homozygous for the previously reported BclI RFLP in the factor VIII gene (1) are heterozygous for the XbaI polymorphism. This new RFLP thus represents a significant addition to available probes for the DNA-based prenatal diagnosis and carrier detection of this disease.

INTRODUCTION

Hemophilia A (classic hemophilia) is the most common severe inherited bleeding disorder, affecting at least one male in 10,000. The disease is caused by a deficiency of factor VIII, an essential protein cofactor in the intrinsic coagulation pathway. The gene for factor VIII is located at the distal end of the long arm of the human X-chromosome.

Prenatal diagnosis of hemophilia is possible by fetal blood sampling, but this procedure is performed in the second trimester and carries about a 5 percent risk to the fetus. Carrier detection in women is often equivocal because of the wide range of clotting activity in normal and heterozygous females. Recently, the human factor VIII gene has been cloned and sequenced to completion, and recombinant DNA derived factor VIII has been obtained from transfected tissue culture cells (2,3). The availability of factor VIII DNA probes has led to the identification of several different mutations associated with hemophilia (4-6). A survey of the results supports the prediction that many unique mutations would be found to cause such an X-linked disease. This underscores the necessity of identifying restriction fragment length polymorphisms (RFLP) at this locus for use in DNA-based diagnosis. Such DNA polymorphisms can allow gene tracking in families at

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risk while remaining independent of the particular mutation causing hemophilia.

We initially reported a RFLP in the factor VIII gene involving a BclI site within intron 18 of the gene, comprising a two allele system with frequencies of about 70 percent and 30 percent (1). The predicted frequency of "informative" females heterozygous for this polymorphism is ~42 percent. We demonstrated the applicability of this RFLP in a Southern blot carrier detection and in an antenatal diagnosis using DNA extracted by chorion villus biopsy at eight weeks of gestation (7). This RFLP marker is now in general use. Subsequently, a second RFLP in the factor VIII gene has been reported. A BglI site flanking exon 28 (exact location unreported) is polymorphic, with a reported major allele frequency of from 74 to 94 percent in various populations (5). In combination, these two RFLPs are only informative for about 50-60 percent of females desiring counselling. Two non-factor VIII gene RFLPs, designated DX13 and St14, have been shown to be very tightly linked to the factor VIII locus and provide additional useful markers (1). In particular, St14 has many allelic forms and is almost always informative for gene tracking. However, linked RFLP markers carry the risk of misdiagnosis due to recombination. Even though these two RFLPs are very tightly linked to the hemophilia A locus, there have been several recent reports of recombination between DX13 and factor VIII (8,9), and one report of a cross-over between St14 and the factor VIII gene (10). Hence there is a need to identify further RFLPs within the factor VIII gene itself.

MATERIALS AND METHODS

DNA Preparations and Hybridizations

DNA was prepared from human blood samples as described in (11). Electrophoresis in agarose gels and transfer to nitrocellulose followed standard procedures (12). Probes were gel isolated and labelled with [α-^{32}P]dCTP by calf thymus DNA priming (13) and hybridized in 50 percent formamide, 5xSSC, 50 mM sodium phosphate, 5x Denhardt's solution, 200 µg/ml denatured salmon sperm DNA and 10 percent dextran sulfate at 42°C overnight and washed at 67°C in 0.2xSSC, 0.1 percent SDS before exposure to X-ray film at -70°C with an intensifying screen (12).

Factor VIII Probes and Gene Mapping

Mapping of restriction sites surrounding the polymorphic XbaI site was performed on human factor VIII genomic clones and subclones described in (14). Mapping was performed by standard procedures with single and double

restriction enzyme digests. All map coordinates shown here refer to the coordinates of the factor VIII gene map given in (14). DNA probes were gel isolated and subcloned into appropriate vectors (12) after they were identified as being free of highly repetitious DNA sequences by Southern blot hybridization with labeled, total human genomic DNA.

RESULTS AND DISCUSSION

An XbaI Polymorphism in the Factor VIII Gene

In a screen of factor VIII gene intron and 3'-flanking sequence probes, we found that a 9.6 kb EcoRI fragment from intron 22 hybridized to two alternate XbaI fragments in Southern blots from five individuals. The variant XbaI site was mapped to a location in the gene about 18 kb 3' to the previously reported polymorphic BclI site. An apparent complication with this probe was its hybridization to a second location in the human genome. This was noted with several restriction enzymes. A telling example is the fact that the 9.6 kb EcoRI factor VIII genomic probe fragment hybridizes not only to a 9.6 kb EcoRI, but also to a 21 kb EcoRI fragment in genomic DNA isolated from all individuals tested (Fig. 1, lane 1). Stringent

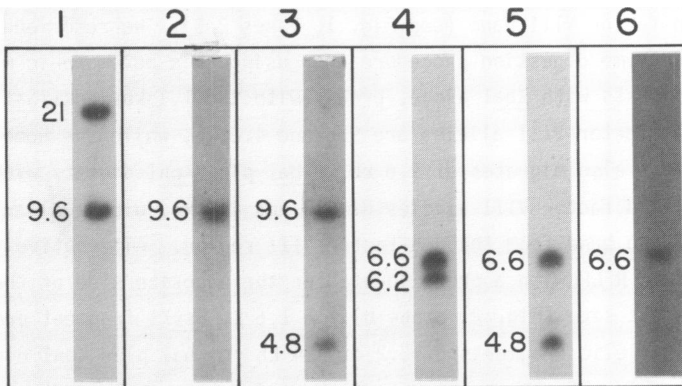

Figure 1. The XbaI RFLP detected by probes in intron 22 of the factor VIII gene. Southern blot hybridization of human genomic DNA samples: Lane 1, EcoRI-digested DNA probed with a 9.6 kb EcoRI fragment of intron 22 demonstrating the two hybridizing regions. Lanes 2-6 are probed with a 1.0 kb EcoRI/SstI fragment of intron 22. Lanes 2 and 3, genomic DNA digested with XbaI showing the large and small polymorphic alleles at 9.6 and 4.8 kb, respectively. Lanes 4 and 5, genomic DNA digested with XbaI plus KpnI showing the large and small alleles at 6.2 and 4.8 kb, while the non-factor VIII hybridizing fragment remains at 6.6 kb. Lane 6, XbaI plus KpnI digested DNA from a hemophiliac (H96; ref. 4) with a gene deletion spanning intron 22. Only the non-factor VIII hybridizing fragment remains.

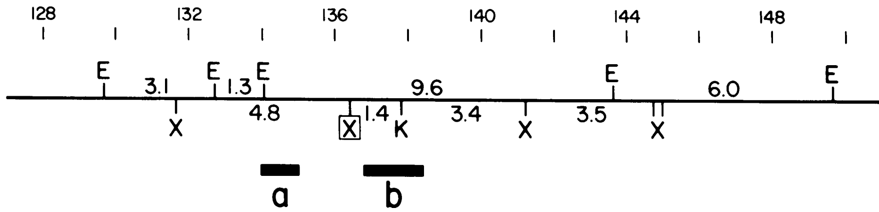

Figure 2. Restriction map of intron 22 of the human factor VIII gene. The size scale in kilobases on the top line corresponds to the numbering scheme for the entire factor VIII gene shown in ref. 14. The size of restriction fragments generated by bordering sites are given (in kilobases) above and below the line. Sites are abbreviated: E = EcoRI, X = XbaI, K = KpnI. The polymorphic XbaI site is boxed in. Probe fragments used to detect the RFLP are shown at the bottom. They are (a) a 1.0 kb EcoRI plus SstI fragment and (b) a 1.6 kb BstXI fragment.

hybridization conditions (68° washes in 0.1X SSC) failed to eliminate this second site of hybridization. Several smaller subfragments of the original 9.6 kb probe still hybridized to this second region. In fact, these two regions are so homologous that several restriction sites are shared. Therefore, we found that double digestion with XbaI and KpnI was helpful in moving the position of the hybridizing polymorphic factor VIII band away from the non-factor VIII band (see Fig. 1, lanes 2-5). We recommend use of this double enzyme digestion procedure when using this polymorphic marker. In genomic digests with XbaI alone, probed with the 1.1 kb EcoRI/SstI fragment, the factor VIII alleles are 9.6 and 4.8 kb, while the homologous region fragment also migrates at 9.6 kb. XbaI plus KpnI digests with the same probe yield factor VIII alleles of 6.2 and 4.8 kb, with a clearly separated 6.6 kb band from the non-factor VIII region. Alternatively, one can detect this RFLP with a probe located on the opposite side of the polymorphic XbaI site (Fig. 2, probe b). A 1.6 kb BstXI fragment probe will hybridize to allelic fragments of 6.2 or 1.4 kb in XbaI plus KpnI digested DNA (see Fig. 3). Of course, either of these fragment probes detect the same XbaI site polymorphism and are suitable choices. Experience has indicated to us that probe b produces autoradiographs that are somewhat superior.

The appearance of a second hybridizing region necessitated further characterization of the intron 22 region. Detailed restriction mapping of isolated factor VIII genomic clones, and the use of several probe fragments in the region of cloned and genomic DNA, contributed to the map shown in Fig. 2 and to the assurance that the polymorphic XbaI site is indeed located in intron 22 of the factor VIII gene. The use of several probes within this

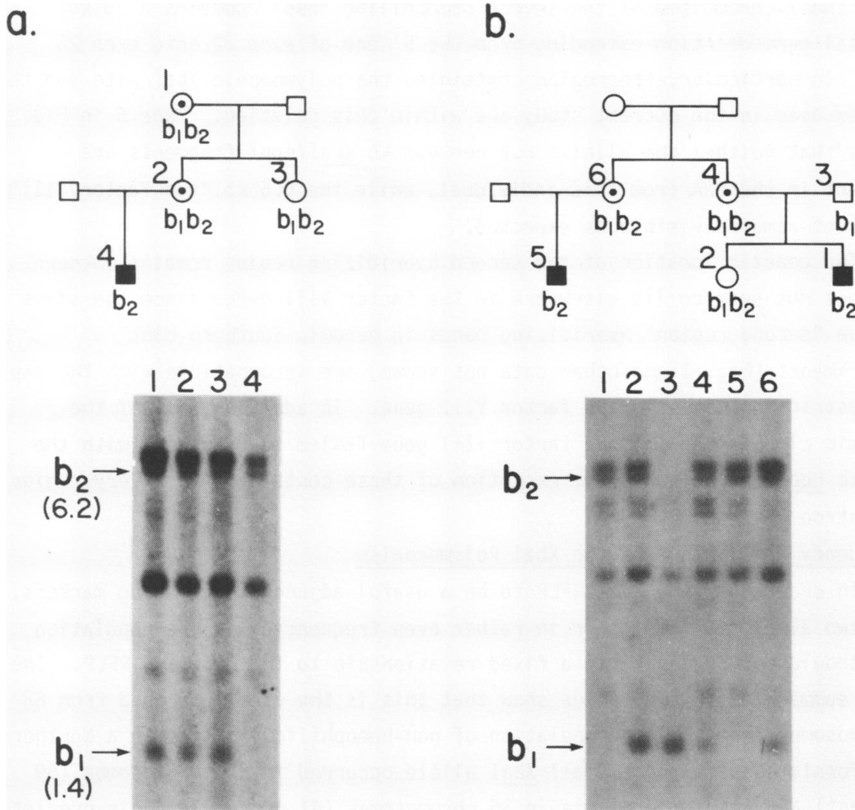

Figure 3. Carrier diagnosis using the XbaI RFLP. Southern blot analysis (below) of individual's DNA corresponds to the pedigrees (above). DNA was digested with XbaI plus KpnI and probed with the 1.6 kb BstXI fragment. b_1 and b_2 refer to the 1.4 and 6.2 kb alleles of the RFLP. In family A, the b_2 allele is associated with the hemophilic factor VIII gene, and the consultand (number 3) is shown to be a carrier who would be informative for prenatal diagnosis. In family B, individual 2 is also diagnosed as a carrier and is a candidate for prenatal diagnosis with the XbaI RFLP. ■ = affected male. ⊙ = female carrier (known before RFLP analysis). Consultands in each family whose carrier status was unknown before RFLP analysis are indicated as O . (The hybridizing band above the b_2 allele is the 6.6 kb fragment from the non-factor VIII hybridizing region, and the 3.4 kb hybridizing band is from the factor VIII gene by reference to Fig. 2. Individual 3 in family B is also missing the 6.6 kb, non-factor VIII fragment.)

region revealed hybridizing fragments consistent with that map and with the assignment of the DNA sequence polymorphism at that location. Further confirmation was acquired by Southern blot analysis of DNA known to be deleted in this portion of the factor VIII gene. We had previously reported

that the X chromosome of the severe hemophiliac "H96" contains a 39 kb partial gene deletion extending from the 5' end of exon 22 into exon 25 (4). In particular, the region containing the polymorphic XbaI site and the probes used in the current study are within this deletion. Lane 6 in Fig. 1 shows that neither the allelic 6.2 nor 4.8 kb XbaI/KpnI fragments are present in the DNA from this individual, while the 6.6 kb "non-factor VIII" fragment remains visible as expected.

The genetic location of the second hybridizing region remains unknown. It does not seem to lie elsewhere in the factor VIII gene, since the sizes of the "second region" hybridizing bands in genomic Southern blot experiments (Fig. 1 and other data not shown) are incompatible with the map of restriction sites in the factor VIII gene. In addition, all of the genomic clones spanning the factor VIII gene failed to hybridize with the 9.6 kb EcoRI probe, with the exception of those containing this very region of intron 22.

Frequency and Linkage of the XbaI Polymorphism

In order for this XbaI RFLP to be a useful adjunct to existing markers, the two alleles should occur in rather even frequencies in the population and should not segregate in a fixed relationship to the existing RFLP. The data summarized in the tables show that this is the case. In data from 88 chromosomes from a mixed population of non-hemophilic visitors to a Northern California hospital, the small XbaI allele occurred in 52 chromosomes (59 percent) and the large allele in 36 chromosomes (41 percent). This predicts that about 48 percent of females seeking counselling will be heterozygous for the marker. Furthermore, Table 2 shows that the XbaI RFLP can be useful in cases in which the BclI polymorphism is not. The small BclI allele (a_1) was found to be associated with either XbaI allele b_1 or b_2. Of the four possible haplotypes, no examples were found of the a_2b_1 combination--large BclI plus small XbaI. It is not surprising to find some degree of linkage disequilibrium between two such close markers, and it is often the case that one of the four possible haplotypes will not be found in a survey of this size. The two polymorphic restriction sites lie 18 kb apart, which roughly corresponds to a predicted recombination frequency of only 0.0002. The tabulated data suggest that the base change(s) producing the b_1 allele (creation of an XbaI site) occurred in the chromosomal context of the a_1 allele (presence of the polymorphic BclI site) recently compared to the time required for recombination to randomize these two close markers.

TABLE 1. FREQUENCY OF XBAI ALLELIC FORMS IN A POPULATION SAMPLE

	No. of Chromosomes	Percent of Chromosomes
b_1: small fragment	52	59
b_2: large fragment	36	41

DNA samples used were from a set of normal, unrelated individuals of mixed backgrounds.

Despite the degree of linkage disequilibrium between these two factor VIII RFLPs, the tables show that there will be families which are homozygous for the BclI allele a_1 but heterozygous (informative) for the XbaI RFLP (such an example will be shown below). On the basis of Table 2, one can estimate that approximately 25 percent of females will fall into this category, in addition to the ~35 percent who are informative for the BclI polymorphism alone. [From Table 1, the expected frequency of females who are uninformative by virtue of homozygosity of the BclI allele a_1 is .81 x .81 = .66. Of this category, the expected frequency of heterozygotes for the XbaI polymorphism is 2 x .75 x .25 = .375. Hence the added group of informative individuals is expected to be .66 x .375 = .25 of the total.]

Carrier Analysis Using the XbaI Polymorphism

Southern blot analysis of several families shown in Fig. 3 serve to illustrate that the XbaI alleles are inherited in a normal Mendelian fashion and that they are useful in diagnosing the carrier status of individuals in hemophilic families. In family A, individual 3 was a consultand as a possible carrier of hemophilia A. She has a hemophilic nephew and a

TABLE 2. BCLI-XBAI HAPLOTYPE ANALYSIS

Haplotype			
BclI	XbaI	No. of Chromosomes	Percent of Chromosomes
a_1	b_1	45	61
a_1	b_2	15	20
a_2	b_1	0	0
a_2	b_2	14	19

DNA samples are a subset of 74 of the 88 chromosomes in Table 1, as 7 females were doubly heterozygous, thus phases could not be determined. In the chromosomes sampled in ref. 1, the frequency of BclI alleles was 71 percent and 39 percent, while in this ample it is 81 percent and 29 percent.

hemophilic uncle who is not indicated on this pedigree. Her DNA was homozygous, non-informative for the BclI RFLP, but was heterozygous for the XbaI RFLP. In this family, the b_2 allele (6.2 kb) is associated with the hemophilic gene, and the consultand must be a carrier. (These results are consistent with data derived from the linked DX13 RFLP.)

In family B, individual 2 can be diagnosed as a carrier on the basis of this RFLP. She too is heterozygous for the XbaI alleles (but was homozygous for the BclI polymorphism). The males in the family indicate that XbaI allele b_1 associates with the normal factor VIII gene and allele b_2 with the hemophilic gene. Since the consultands in both families are heterozygous for the XbaI RFLP, they would be candidates for prenatal diagnosis using this probe.

We have examined several other families (data not shown) which demonstrate Mendelian inheritance of the XbaI alleles in non-hemophilic families and which demonstrate consistent predictions in families informative for both this and the BclI RFLP. This newly reported restriction fragment length polymorphism in the factor VIII gene should be a useful addition to the available markers to increase the applicability and confidence of DNA based carrier detection and antenatal diagnosis of hemophilia A.

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