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A Plausible Method for the Diagnosis of Genetic Disorders Using Full Length cDNA

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A cDNA of coagulation Factor IX gene has been screened from the λgt11 human fetal liver cDNA library, and used to construct a 2.8-kb full length cDNA after recombining with the N-terminal fragment from pTZ-FIX. Human genomic DNA was isolated, digested with the restriction endonucleases, *Taq*I, *Eco*RI, and *Hin*dIII, and Southern hybridization was performed using the full length factor IX cDNA as a probe. The hybridized bands generated by the restriction endonucleases were the followings: *Taq*I, 0.3, 1.0, 1.6, 1.8, 2.7, 3.7, and 5.3 kb bands; *Eco*RI, 1.8, 4.8, 4.9, 5.5, 6.8, and 12.6 kb bands; *Hin*dIII, 4.1, 4.4, 5.2, 5.8, 7.6, and 12.5 kb bands. When the Southern bands were physically mapped along the genome, about 50-kb continuous region harboring almost all of the genomic region of Factor IX gene was covered. These results suggest a possibility of using an exonal cDNA probe to diagnose abnormalities including large deletions, insertions, and rearrangements along the genome, if there is any.

Key Words: Full length cDNA, Diagnosis, Southern hybridization

INTRODUCTION

Hemophilia B, a coagulation disorder caused by mutations in factor IX gene, occurs primarily in males at a frequency of 1 in 30,000[7]. Since the factor IX gene is located on chromosome X, determination of carrier status in female has been helpful for diagnosis of hemophilia B. Two methods are currently in use to diagnose and analyse hemophilia B. One is measuring the levels of factor IX antigen and clotting activity in the plasma. The accuracy of this method, however, is only about 80% due to the natural variations of these properties among individuals regardless of carrier status[7]. The other method is tracking either a specific mutation site or a closely linked polymorphic marker through an affected pedigree at the DNA level, which is not subject to such variations[2,6]. Direct detection of factor IX mutations has been reported recently using polymerase chain reaction (PCR) and subsequent sequencing[11,20]. Although this technique confers the ability to identify a specific mutation site unambiguously, it requires laborious and time-consuming work, including amplification and concomitant sequencing of the total region of the gene consisting of eight exons and long introns. Therefore, it is necessary to narrow the target region along the gene before localized amplification and sequencing.

In this study we adapted the Southern hybridization method to detect DNA polymorphisms and defects such as large deletions, insertions, and rearrangements. Unlike the PCR technique, Southern blot analysis provides the possibility of analysing mutations over a large region of the gene at once, including whole exons and introns as well as untranslated regions (UTRs), depending on the probe. Therefore the probe and restriction endonucleases used for a particular experiment are critical. We describe here the isolation of the entire 2.8 kb length of factor IX cDNA in order to diagnose possible mutations present over the entire factor IX gene including whole UTRs. We performed genomic Southern blot analysis with normal human genomic DNA using the full length cDNA as a probe. The sizes of the DNA fragments digested with the restriction endonuclease and subsequently hybridized were the same as those which we have expected based upon the nucleotide (nt) sequences published[21]. This result led us to propose a simple and fast method to diagnose mutations in the factor IX

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gene including exons, introns, and UTRs concurrently. The method described here is widely applicable to detect gross defects in any gene of interest, given a suitable gene probe.

MATERIALS AND METHODS

1. Screening of the Liver cDNA Library for Isolation of Human Factor IX

A human fetal liver cDNA library (Dr. M. Yoo, Keimyung Univ., Taegu, Korea) was used to screen coagulation factor IX cDNA according to the protocols suggested by Clontech company with slight modifications. Briefly, about 30,000 plaques infected into Y1088 host cells (ATCC) were plated onto the 150-mm plate, and phage DNAs were transferred into the Nylon membrane filter (Hybond, Amersham). The filters were pre-hybridized in hybridization solution containing 5 x SSC, 1% blocking reagent (BM), 0.1% N-laurylsarcosine and 0.02% sodium dodesyl sulfate (SDS) for 4 h and then hybridized with a α-^{32}P-dCTP labeled 730 bp DNA fragment from 24F9 factor IX cDNA[12]. After being washed, the filters were dried and autoradiographed.

2. Isolation of Recombinant λ DNA

Positive plaques were picked and dissolved in 1 ml of SM buffer with 20 μl of chloroform at 4℃ overnight. This lysate was used for further amplification by infecting Y1088 bacterial host cells in liquid broth following the protocols described in Molecular Cloning[13].

3. Construction of Full Length Factor IX cDNA

To construct full length factor IX cDNA, p2.4FIX containing the 3' portion of factor IX cDNA as well as the long 3'-UTR was digested with *Hin*dIII and *Ava*I. The 2.1 kb *Hin*dIII-*Ava*I fragment was isolated and ligated with the 3.56 kb *Hin*dIII-*Ava*I fragment of pTZ-FIX[12] harboring the pTZ vector as well as the 5' portion of factor IX cDNA to generate the pTZ-2.8FIX (Fig. 2).

4. Genomic DNA Preparation

Genomic DNA was extracted from leukocytes by modifying the procedure described by Maniatis *et al*[13]. Briefly, 5 ml of EDTA-anticoagulated peripheral blood was diluted with the same volume of PBS to lyse cells and centrifuged at 3,500 *g* for 15 min. The pellet was resuspended in 10 ml of PBS and centrifuged again. This procedure was repeated three times until a pale pellet was obtained. The pellet, resuspended in 5 ml of DNA extraction buffer (10 mM Tris-Cl, pH 8.0, 0.1 M EDTA, 0.5% SDS), was treated with proteinase K for 16 h at 37℃, and extracted with phenol three times and then precipitated with ethanol.

5. Southern Blot Hybridization

Genomic Southern blot hybridization was performed following the procedure invented by Southern[18] with slight modifications. Ten micrograms of human genomic DNAs digested with appropriate restriction enzymes (*Taq*I, *Bam*HI, *Hin*dIII) were separated on a 0.8% agarose gel at 20~30 volts overnight. DNAs on the gel were depurinated in a solution containing 0.25 N HCl for 10 min, and denatured in 0.5 N NaOH and 1.5 M NaCl twice for 15 min each. DNAs were neutralized in a solution containing 0.5 M Tris-Cl (pH 7.5) and 3 M NaCl twice for 15 min each at room temperature, and then transferred to a nylon membrane (Schleicher & Schuell NYTRAN NY 13 M). After DNAs were fixed by UV irradiation, the membrane was prehybridized in 10 ml of prehybridization buffer containing 5x SSC, 1.0% (w/v) blocking reagent, 0.1% N-laurylsarcosine, 0.02% SDS for 3 h at 65℃. Hybridization was performed in the same solution with α-^{32}P-dCTP labelled factor IX cDNA probe for 12~16 h at 65℃. Membranes were washed with 2x SSC containing 0.1% SDS, and then with 0.2x SSC containing 0.1% SDS for 30 min at 65℃, sequentially. The blot was visualized by autoradiography for 48 h at -70℃ using Kodak XK-1 X-ray film with an intensifying screen.

RESULTS

1. Isolation of Human Factor IX cDNA by Screening Liver cDNA Library

Human factor IX gene is expressed in hepatocytes as a 2.8 kb-long mRNA, of which 1.4 kb are not translated[1]. In order to isolate the full length cDNA, we screened the human fetal liver cDNA library through plaque hybridization using a cloned 1.4 kb factor IX cDNA as a probe[12]. From 9 x 10^4 plaques screened, 4 positive clones were isolated and the DNAs were purified and analyzed on a 0.8% agarose gel after digestion with *Eco*RI restriction endonuclease (data not shown). Since the cDNA had been inserted into the *Eco*RI site of the lambda vector, the size of the inserted

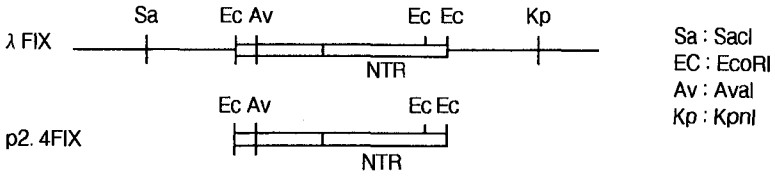

Fig. 1. Schematic drawing of λFIX and p2.4FIX containing 2.4 kb cDNA of human factor IX gene missing the 5' portion of the gene. Rectangle represents the cDNA and the UTR stands for the 1.4 kb long 3' nontranslated region of factor IX cDNA.

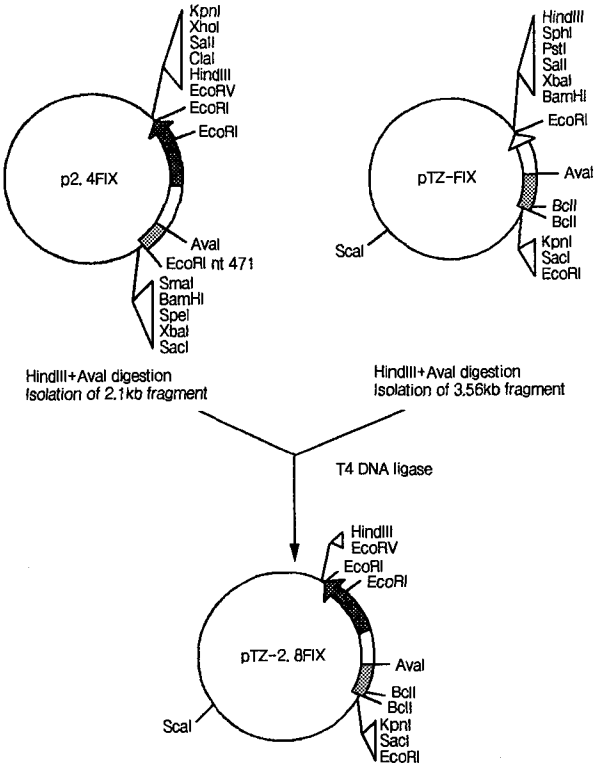

Fig. 2. The construction scheme for pTZ-2.8FIX.

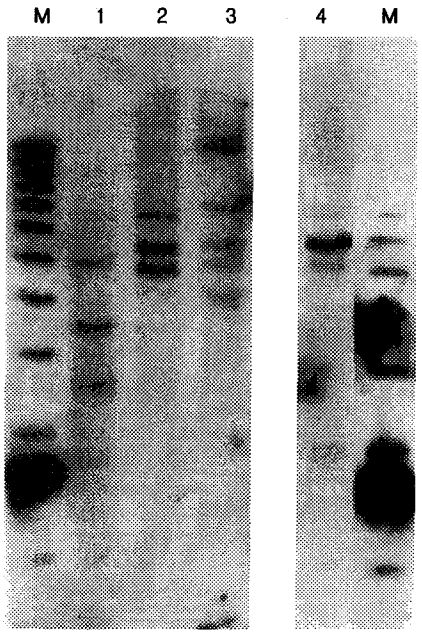

Fig. 3. Southern blot analysis of genomic DNA isolated from normal person. Lane M, 1 kb ladder marker; Lane 1, *Taq*I digested genomic DNA; Lanes 2 and 4, *Eco*RI digested genomic DNA; Lane 3, *Hin*dIII digested genomic DNA.

DNA can be calculated based upon the size of the *Eco*RI restriction fragment. The recombinant phage harboring the longest factor IX cDNA insert was selected and named λFIX (Fig. 1). As shown in Figure 1, the cDNA in λFIX contains an internal *Eco*RI recognition site at the 3' UTR as well as a single *Ava*I site near the amino-terminus, and the total length has been proved to be 2.4 kb missing the 470 bp of the amino terminus of factor IX cDNA after sequencing both junction areas (data not shown).

2. Construction of the Plasmid Containing Full Length Factor IX cDNA

To construct the full length factor IX cDNA, 2.4 kb DNA was isolated from λFIX after partial *Eco*RI digestion and sub-cloned into the plasmid pBluescript KS(+), generating p2.4FIX. And then, the 2.1 kb *Hin*dIII-*Ava*I fragment from p2.4FIX containing the 3' portion of factor IX cDNA including the long 3'-UTR and the 3.56 kb *Hin*dIII-*Ava*I fragment of pTZ-FIX[12] harboring the pTZ vector as well as the 5' portion of factor IX cDNA was isolated, and ligated to generate the pTZ-2.8FIX (Fig. 2). The full length intact 2.8 kb factor IX cDNA was isolated and used as an exonal probe for genomic Southern blot analysis, after double digestion of pTZ-2.8FIX with *Hin*dIII and *Kpn*I.

3. Analysis of Genomc DNA by Southern Blot Hybridization Using Full Length Factor IX cDNA as a Probe

To test whether the hybridization method is useful for diagnosis we digested normal human genomic DNAs with *Taq*I, *Eco*RI or *Hin*dIII, and did Southern analysis with the exonal probe: *Taq*I generated 0.3, 1.0, 1.6, 1.8, 2.7, 3.7, and

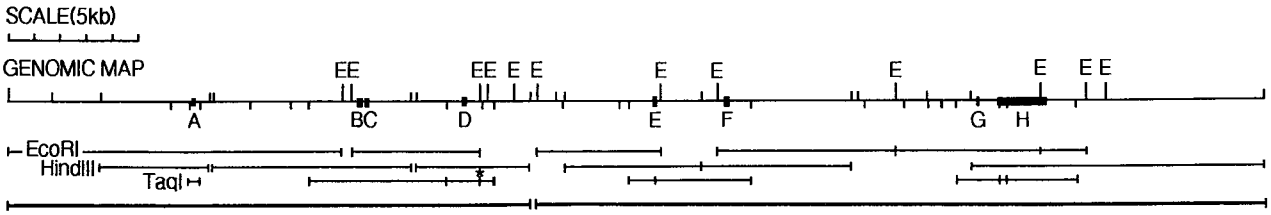

Fig. 4. Schematic diagram of the factor IX gene showing restriction enzyme sites. E with vertical line above the genomic map stands for the *Eco*RI recognition site. The short vertical lines above and below the genomic map indicate the *Hin*dIII and *Taq*I recognition sites, respectively. Eight black boxes represent eight exons which are designated as capital letters from A through H. The horizontal lines at the bottom represent Southern bands generated with each restriction endonucleases by hybridization with the 2.8 kb exonal probe. A known *Taq*I polymorphic site is marked as *. The bald horizontal line represents the genome coverage with regard to the combinatorial results of above three restriction endonucleases; the 300 bp missing portion from *Hin*dIII (nt 12,936) to *Eco*RI (nt 13,236) site located in the fourth intron was indicated as blank along the bald line.

Table 1. Expected sizes of restriction enzyme fragments in normal control

Enzymes	Sizes in normal (kb)
*Taq*I	0.3, 5.3, 1.8[a], 1.0, 3.7, 1.6, 2.7
*Eco*RI	12.6, 4.9, 4.8, 6.8, 5.5, 1.8
*Hin*dIII	4.1, 7.6, 4.4, 5.2, 5.8, 12.5[b]

[a] Contains a polymorphic *Taq*I site, therefore certain individual could contain a 1.3 kb (see Fig. 1)
[b] This size has been deduced from the band size of the largest fragment (See Fig. 3, lane 3) containing exons G and H (Fig. 1).

5.3 kb bands; *Eco*RI generated 1.8, 4.8, 4.9, 5.5, 6.8, and 12.6 kb bands; *Hin*dIII generated 4.1, 4.4, 5.2, 5.8, 7.6, 12.5 kb bands (Fig. 3 and Table 1). In the case of *Taq*I, a 1.37 kb band instead of a 1.8 kb could be generated after blotting, since there is one polymorphic *Taq*I site at nucleotide 11,111. The nucleotide number is based upon the numbering of Yoshitake *et al*[21]. This site is known to be less polymorphic among Asians[8]. Except for this, all enzyme sites tested here were intact (Fig. 4). Thus, these results presented a possibility of using an exonal cDNA probe to diagnose abnormalities such as deletions, insertions, and rearrangements in hemophilia B patients. If there are such alterations in the genomic sequences in factor IX gene, the new bands will appear along with the unalterred bands.

DISCUSSION

Either gross gene abnormalities or a few nucleotide changes in critical regions of genes cause genetic diseases, and several methods are applied to detect such mutations. Mutational analysis based on molecular biology is well-developed and is used for analysis of genetic diseases and prenatal diagnosis. Point mutations can be analyzed either by restriction endonuclease analysis such as restriction fragment length polymorphism (RFLP), which recognizes the mutation that abolishes or creates a recognition nucleotide sequence for a particular enzyme, or by rapid amplification of particular regions of the gene using PCR and subsequent sequencing[11,20], or single-strand conformational polymorphism (SSCP)[4]. However, analyzing point mutations is still time consuming and require many laborious steps. Therefore, it is reasonable to narrow the mutation point through Southern blotting using an appropriate probe[9,19]. In the case of hemophilia B, researchers have used at most about a 2.0 kb cDNA probe missing the 3' untranslated region for diagnosis. Recent studies on the UTR have revealed that this region could be involved in the transcriptional and/or translational control of the related gene[10,14,17]. Therefore, we have included UTR for mutational analysis. As shown in Figure 4, a 2.8 kb factor IX cDNA probe for the Southern blot could reveal abnormal gene defects if present along the 35 kb factor IX gene in the human genome. In this particular experiment, the probe actually covers about 50 kb, missing only 300 bp from *Hin*dIII (nt 12,936) to *Eco*RI (nt 13,236) (Fig. 4) located in the fourth intron between exons D and E. By choosing different combinations of restriction endonucleases, such as *Bgl*II, this 300 bp gap will be filled in. Since we know the total genomic DNA sequences of the factor IX gene[21], we can choose the appropriate restriction enzymes to resolve the region of factor IX DNA of interest. Although Southern blot method has some difficulties in detecting either small alterations such as a few base deletions or insertions or point mutations, this limitation could be overcome by applying denaturing gradient-gel electrophoresis and SSCP[3,5,15,16]. The benefit of this Southern blot analysis over other techniques is that by simply selecting

several restriction enzymes and combining the results of Southern band patterns we can detect genetic abnormalities not only in coding regions but also in introns as well as in the vicinities of the gene.

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