RFLPs OF FACTOR IX GENE IN JAPANESE HAEMOPHILIA B FAMILIES AND GENE DELETION IN TWO HIGH-RESPONDER-INHIBITOR PATIENTS

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Summary   The factor IX genes in four Japanese families with haemophilia B were analysed for the restriction fragment length polymorphisms (RFLPs) of *Taq*I, *Xmn*I and *Dde*I, using subcloned intragenic DNA fragments as probes (probes VIII and XIII).   The factor IX genes in 12 patients with haemophilia B and three high-responder-inhibitor cases showed no size difference using a cDNA probe (cVII) when restricted by *Taq*I, *Eco*RI and *Hind*III.   Complete gene deletions were observed in two other high-responder-inhibitor cases.

INTRODUCTION

The gene for coagulation factor IX has been identified and completely sequenced by Choo *et al.* (1982), Anson *et al.* (1984), Kurachi and Davie (1982), Yoshitake *et al.* (1985) and Jaye *et al.* (1983).   Also, the common polymorphic sites in the normal factor IX gene have been clarified (Yoshitake *et al.*, 1985; Camerino *et al.*, 1984; Giannelli *et al.*, 1984; Winship *et al.*, 1984), to which pathogenic genes for haemophilia B were detected to be linked in a number of families using subcloned DNA fragments as probes and a cDNA sequence.   These restriction fragment length polymorphisms (RFLPs) are now used for the female carrier detection and the prenatal diagnosis (Camerino *et al.*, 1984; Giannelli *et al.*, 1984; Winship *et al.*, 1984; Camerino *et al.*, 1985; Peake *et al.*, 1984; Hay *et al.*, 1986; Grunebaum *et al.*, 1984; Brocker-Vriends *et al.*, 1985).   Although the abnormal factor IX genes in haemophiliacs have been analysed by the several investigators, a partial or complete deletion of the genes was found mainly in the inhibitor patients (Giannelli *et al.*, 1983; Hassan *et al.*, 1985a, 1985b; Bernardi *et al.*, 1985).

This paper described a study of the RFLPs in 10 Japanese families with haemophilia B, using subcloned intragenic DNA fragments of the factor IX gene as probes.

Also, the restriction fragment length in 14 patients with haemophilia B, including five inhibitor cases were observed.

MATERIALS AND METHODS

Subjects. Eleven haemophilia B patients from 10 unrelated families, all of whom were treated in our clinic, consisted of six patients with severe form, two with the moderate or mild form, one with Bm form and two with inhibitor (allo-antibody) type. The two inhibitor cases were high responders with more than 50 Bethesda units, one of whom had a temporary inhibitor with a peak of 78 Bethesda units that subsequently disappeared even when factor IX preparation was administered. The family members tested included the propositus, other patients if they existed, and the propositus' parents. All mothers were detected as definite carriers according to their family pedigrees and/or their haemostatic findings. Their DNAs were used for RFLPs analysis using subcloned DNA probes as described below.

DNAs from the 11 patients and three other inhibitor cases, were studied for the detection of abnormal restriction sites in their factor IX genes using a cDNA probe. These three inhibitor cases were all high-responders.

Gene probes. Both the subcloned factor IX gene probes VIII and XIII and the cDNA probe cVII provided by Prof. Brownlee of Oxford University were described by Giannelli *et al.* (1984) and Winship *et al.* (1984). The probes VIII, XIII and cVII were cloned into the *Pvu*II site of ampicillin-resistant plasmid vectors pATs, and were stabbed in *E. coli* MC1061 that were cultured in media with ampicillin. After chloramphenicol amplification, the plasmids were separated by sucrose centrifugation. Then the DNAs from the plasmids were separated by ethidium bromide-cesium chloride centrifugation. After the excision of plasmids DNAs with the appropriate restriction enzymes described in Table 1, factor IX probes were separated by agarose gel electrophoresis. The probes VIII, XIII and cVII were 2.5, 0.46 and 2.0 kb in length, respectively. They were radiolabeled by nick translation (Rigby *et al.*, 1977), and were denatured by heating before hybridisation.

RFLPs analysis. Whole citrated-blood, 10 to 20 ml, were frozen at $-70°C$. After centrifugation, the precipitates were treated with SDS and proteinase K by the method of Grunebaum *et al.* (1984). Then DNA was obtained by repeated phenol-chloroform extractions.

Table 1. Subcloned intragenic DNA probes (VIII and XIII) and cDNA probe (cVII) for factor IX gene.

Probes	Length	Plasmid vectors	Stabbers	Excision enzymes
VIII (subclone)	2.5 kb	pAT 153/*Pvu*II/8	MC 1061	*Eco*RI/*Hind*III
XIII (subclone)	0.46 kb	pATX	MC 1061	*Bam*HI/*Hind*III
cVII (cDNA clone)	2.0 kb	pAT 153/*Pvu*II/8	MC 1061	*Bam*HI/*Hind*III

DNA samples were then submitted to reaction with an appropriate restriction enzyme. *Taq*I, *Xmn*I and *Dde*I were used for RFLP analysis in the families using probes VIII and XIII, and *Taq*I, *Eco*RI and *Hin*dIII were used for detection of the restriction fragment length in the patients' DNA using probe cVII. After ethanol
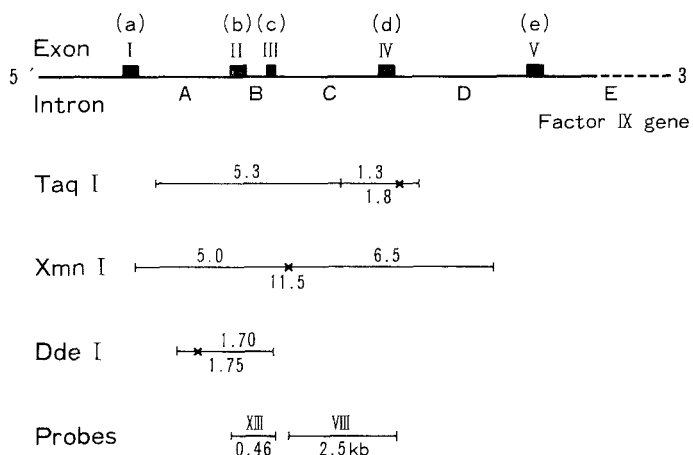

Fig. 1. Three common polymorphic sites of factor IX gene and hybridisation sites of the probes VIII and XIII.

Table 2. Method of RFLP analysis of factor IX gene.

(Whole blood)

Freezing at −40°C

Centrifuging

SDS and proteinase K treatment

Repeated phenol-chloroform extraction
↓
(DNA)

Restriction enzyme treatment

{ *Taq*I, *Xmn*I, *Dde*I for probe VIII, XIII
{ *Taq*I, *Eco*RI, *Hin*dIII for probe cVII

Ethanol extraction
↓
(Restricted DNA)

Agarose gel electrophoresis with SDS

Denaturation by 0.5 M NaOH

Southern blotting to nitrocellulose filter
↓
Prehybridisation

Hybridisation with ^{32}P probe VIII, XIII or cVII

Autoradiography

precipitation and SDS treatment, samples were electrophoresed in 0.7% agarose using a submarine apparatus. Then the DNA fragments in the gel were denatured by 0.5 M sodium hydroxide, and transferred onto a nitrocellulose membrane filter by Southern blotting (Southern, 1975). After prehybridisation, the filter was hybridised with each radiolabeled gene probe, and used for autoradiography (Table 2).

RESULTS

RFLPs in the carriers restricted by TaqI, XmnI or DdeI

*Taq*I, *Xmn*I and *Dde*I polymorphisms in the two factor IX genes in the carriers of 10 haemophilia B families, were observed. All carriers were diagnosed by either their family pedigrees or by their haemostatic findings.

*Taq*I restriction is a typical way to detect a dimorphism of the gene, but only
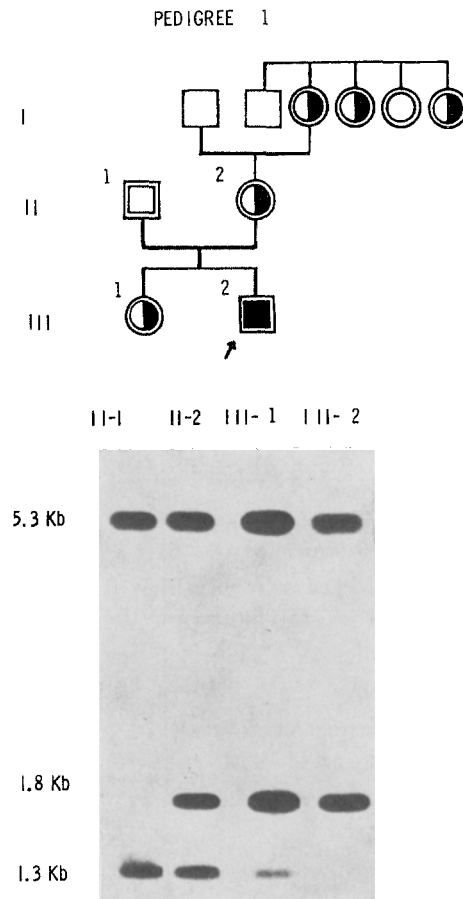

Fig. 2. *Taq*I RFLP in a haemophilia B family.

one carrier out of 10 showed dimorphism of fragment length.   In the *Xmn*I restriction of the gene, the DNAs from two carriers out of the remaining nine, and in *Dde*I restriction, one carrier out of the remaining seven showed dimorphisms.   Although Giannelli *et al.* (1984) and Winship *et al.* (1984) reported that approximately 60% of haemophilia B genes could be detected in the families by observation of these three polymorphic sites, only four families out of 10 were informative by RFLP analysis of their factor IX genes in our observations.

TaqI RFLP in a haemophilia B family

A *Taq*I RFLP in a family is shown in Fig. 2.   The two genes in the mother (who was the carrier having 40% IX:C plasma activity), showed a dimorphism of $5.3 + 1.8$ and $5.3 + 1.3$ kb, and the gene in the propositus (who has 1–3% plasma IX:C activity and 11% IX antigen), was $5.3 + 1.8$ kb.   Therefore the $5.3 + 1.8$ kb gene was detected as the pathogenic gene.   And since the gene in the father showed $5.3 + 1.3$ kb, the sister of the propositus, whose genes consisted of $5.3 + 1.3$ from the father and $5.3 + 1.8$ which was the pathogenic gene from the mother, was considered to be a carrier.   Her clotting activity and antigen of factor IX were both approximately 40%.
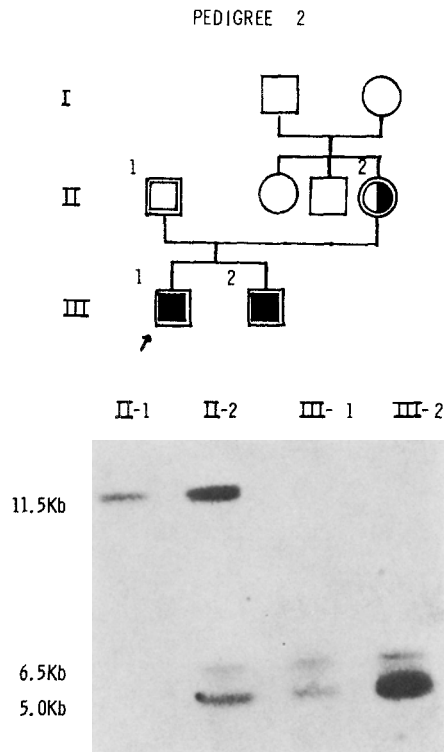
PEDIGREE 2

Fig. 3.   *Xmn*I RFLP in a haemophilia B family.

XmnI and DdeI RFLPs in haemophilia B families

In *Xmn*I restriction of a family, DNA from the mother showed a dimorphism of 11.5 and 6.5 + 5.0 kb by probes VIII and XIII. Since the propositus and the brother who was also a patient of severe form, showed two small restricted bands of 6.5 + 5.0, this gene was determined as the pathogenic gene (Fig. 3).

In the *Dde*I restriction of another family, the genes from the mother showed 1.75 and 1.70 kb. The former had a 50 bp insertion. Since the propositus had 1.70 kb gene, it was the pathogenic gene. The genes from the sister who was already detected as a carrier according to her haemostatic findings, consisted of the abnormal gene of 1.70 kb from the mother and the normal gene of 1.75 kb from the father. The brother who was not a patient, had the normal gene of 1.75 kb from the mother (Fig. 4).

TaqI restriction of the patient genes

In order to observe either deletions of abnormalities of the restriction fragments of factor IX gene in the patients, DNAs from all patients were restricted by enzymes and their fragment length were detected using the cDNA probe cVII. This probe runs from residues 17 to 2,002 of the factor IX cDNA, so as to hybridise with the amino acid-coding exons. A total number of 14 patients including five inhibitor cases, was tested.

In the *Taq*I restriction, several positive bands consisting of three major bands of 3.5, 2.6 and 1.7 kb were observed in both normal and patient DNAs except two patients as is shown in Fig. 5. Two inhibitor cases with high titers and one temporary inhibitor case were contained in these band-positive patients. The length
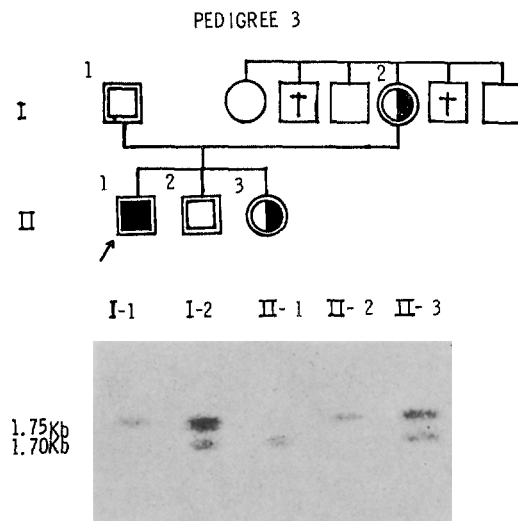

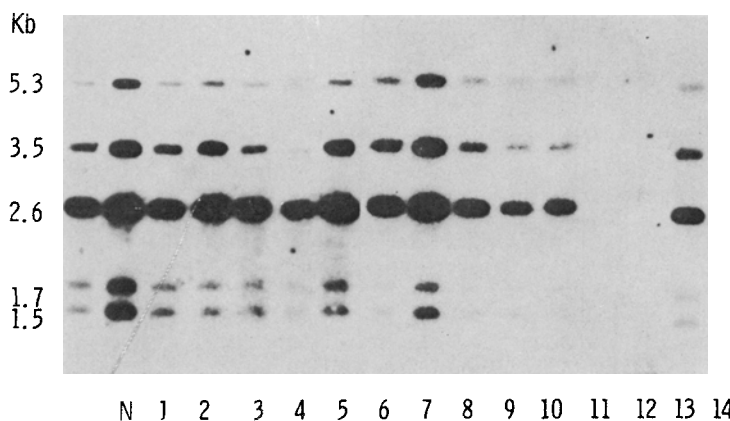Fig. 4.   *Dde*I RFLP in a haemophilia B family.

Fig. 5.   Fragment length of factor IX genes restricted by *Taq*I in 14 haemophilia B patients.
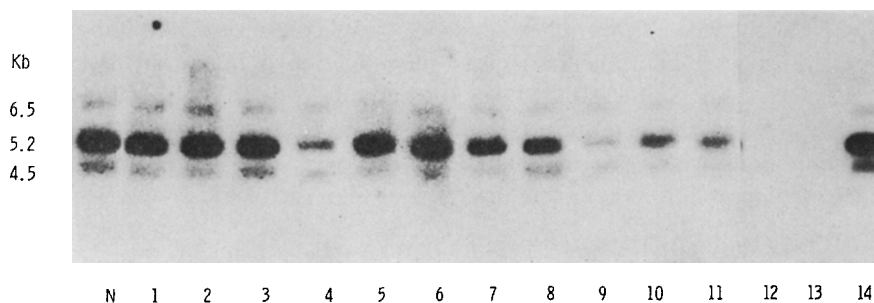

Fig. 6.   Fragment length of factor IX genes restricted by *Eco*RI in 14 haemophilia B patients.

of the positive fragment bands in the 12 patients was the same as that in normal subjects, and corresponded to the sizes reported by Hassan *et al.* (1985a).   Though we can expect to find size differences if any partial deletion or mutation occurred in the *Taq*I TCGA site which includes a hot spot of mutation, no abnormality of the fragment length could be observed in comparison with those in the normal gene.

In the remaining two inhibitor cases who were both high responders, no positive bands were detected with probe cVII hybridisation.

EcoRI restriction of the patient genes

As the next restriction enzyme for patient genes, we used *Eco*RI which attacks G/AATTC.   As can be seen in Fig. 6, three major fragments of 6.5, 5.2 and 4.5 kb nucleotides were observed except in two inhibitor cases.   These fragment sizes of the gene were similar to those reported by Hassan *et al.* (1985b), and no discrepancy of length was observed between the 12 tested subjects.   In two inhibitor cases, no bands were detected as was found after *Taq*I digestion.
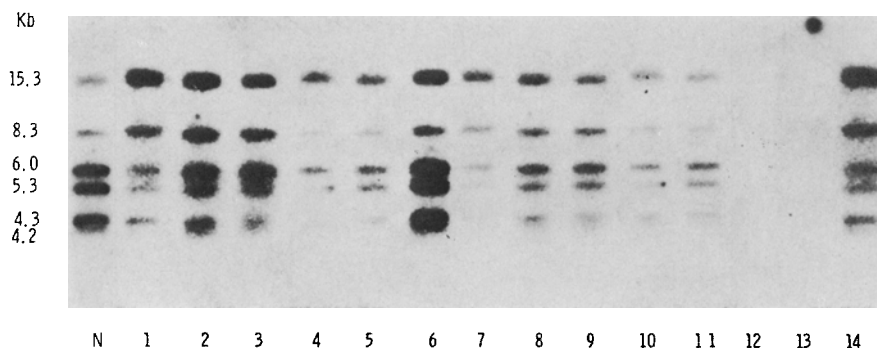
Fig. 7. Fragment length of factor IX genes restricted by *Hin*dIII in 14 haemophilia B patients.

HindIII restriction of the patient genes

As a final treatment with restriction enzyme, *Hin*dIII was chosen to attack the A/AGCTT sequences. Several hot bands were observed on the film in both normal and patient genes except the previously mentioned two inhibitor cases, showing 15.3, 8.3, 6.0, 5.3, 4.3 and 4.2 kb from the cathodal side. No size difference was detectable between the band-positive subjects. In the two inhibitor cases, no hybridisation bands were seen in *Hin*dIII restricted genes (Fig. 7). Consequently, these two patients showed complete deletions of their factor IX genes.

DISCUSSION

The three common polymorphic sites of the factor IX gene are now thought to be very useful for the detection of the pathogenic gene in haemophilia B families (RFLP) (Yoshitake *et al.*, 1985; Camerino *et al.*, 1984; Winship *et al.*, 1984). Many reports used for this purpose subcloned DNA and cDNA probes (Camerino *et al.*, 1984; Giannelli *et al.*, 1984; Winship *et al.*, 1984; Camerino *et al.*, 1985; Peake *et al.*, 1984; Hay *et al.*, 1986; Grunebaum *et al.*, 1984; Brocker-Vriends *et al.*, 1985). A *Taq*I site (T/CGA) in intron D is a typical polymorphic site (Yoshitake *et al.*, 1985; Giannelli *et al.*, 1984), and one of the hot spots of mutations (Barker *et al.*, 1984). Though approximately 25% of the two factor IX genes in women are reported to have dimorphism in this site, only one carrier out of 10 showed it in our observation. All the remaining carriers showed a monomorphism of $2 \times (5.3$ and 1.8 kb). In this family, the pathogenic gene which showed 5.3 and 1.3 kb fragments must be inherited from the mother of the propositus. The sister of the propositus who was detected as a carrier according to her haemostatic findings, proved to have two factor IX genes consisting of one abnormal gene of 5.3 and 1.3 kb from the mother and the other normal gene of 5.3 and 1.8 kb from the father. Since the propositus showed 1–3% activity but 11% antigen, it seems difficult to perform prenatal diagnosis by the conventional method.

In *Xmn*I and *Dde*I RFLP analysis, the rates of dimorphisms in the carriers were also lower in our observation than those reported by Winship *et al.* (1984), and the fragment length of the genes in the most of carriers showed $2 \times (11.5 \text{ kb})$ by *Xmn*I and $2 \times (1.70 \text{ kb})$ by *Dde*I in contrast with the result reported by Winship *et al.* (1984). Since only a small number of carriers were tested in this observation, it will be necessary to examine the frequency of the polymorphic alleles in a much larger samples of the Japanese population before predicting the usefullness of these RFLP's. We must make efforts to search appropriate enzymes and DNA markers for RFLPs of Japanese factor IX genes.

In regard to the abnormalities of the factor IX genes in haemophilia B patients, either a partial or a total deletion of factor IX gene was reported in inhibitor cases (Giannelli *et al.*, 1983; Hassan *et al.*, 1985a, 1985b; Bernardi *et al.*, 1985) except in one family (Chen *et al.*, 1985). However all of the inhibitor cases do not show a detectable gene deletion. In our observations, the factor IX genes in two inhibitor cases out of five showed a complete deletion, whereas the remaining three cases had the total gene. They were all high responders, though one of them was a temporary inhibitor case.

Point mutations of the genes in haemophilia B patients were reported in the CRM-positive variant form, for example, factor IX Chapel Hill (Noyes *et al.*, 1983) and factor IX Alabama (Davis *et al.*, 1984). Restriction enzymes such as *Taq*I are a useful marker to search for a point mutation, and several point mutations were reported in haemophilia A patients detected by the method of RFLP search (Antonarakis *et al.*, 1985; Gitscher *et al.*, 1985; Gitscher *et al.*, 1986). In haemophilia B genes, a definitive mutation point has not been found by screening using restriction enzymes. However a mutation in a splice junction was determined by the analysis of DNA sequence (Rees *et al.*, 1985). In our research, *Taq*I, *Eco*RI and *Hind*III were used for reaction with the genes of 12 patients, and no abnormal fragments were detected. It may be required to observe size difference of the fragments using other enzymes.

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