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Detection of known haemophilia B mutations and carrier testing by microarray

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Summary

The molecular basis of haemophilia B is heterogeneous and many mutations of the Factor IX (FIX) gene have been characterised. Using the allele-specific arrayed primer extension (AS-APEX) technology, we have designed a FIX array to simultaneously analyse 69 mutations found in British, Thai and Chinese patients. This technology overcomes the problem of multiple reverse dot-blot analysis and has a 100% accuracy in the detection of both affected subjects and carriers in families with known mutations. In seven unknown mutations from Thailand, the array could detect the specific mutation in five and in the remainders the normal primer at specific spots failed to extend due to a mutation a few nucleotides upstream, thus allowing their identification. Hence this FIX array can detect 53% of the 2891 mutation entries in the FIX database. Each of the microarray slide can be used for three different test samples and would be useful for carrier testing for common mutations and prenatal diagnosis. It is simpler and more cost effective than genome sequencing and would be particularly useful in laboratories with limited technical capabilities.

Keywords

Haemophilia B, FIX microarray, AS-APEX, FIX mutations

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Introduction

Haemophilia B (Christmas Disease) is an X-linked coagulopathy due to deficiency of the clotting factor IX (FIX) (1). It affects approximately 1 in 25,000 – 30,000 live-born males (2), usually females with one defective gene are asymptomatic carriers. The molecular basis of the disease is heterogeneous and over 900 mutations have been characterised (3). While a number of restriction fragment length polymorphisms (RFLPs) can be used for linkage of the affected gene in Caucasians (4), in Orientals, who lack heterozygosity for these RFLP sites (4–6), carrier detection and prenatal diagnosis are mainly performed by direct mutation detection (5).

The development of microarray technology (7) has enabled large-scale sequence analysis or mutation detection. Employing the allele-specific arrayed primer extension (AS-APEX) technology, we have recently devised a microarray for simultaneous analysis of multiple mutations of the α- and β-globin genes (8). We now apply the same technology for the manufacture of a microarray for detecting FIX mutations in known mutants from three populations as well as those that had been recorded three or more times on the FIX database. This microarray should be particularly useful for detection of common mutations, carrier testing in family members of known index patients and in subsequent prenatal diagnosis.

Materials and methods

Genomic DNA were prepared from peripheral blood leucocytes of 69 unrelated Haemophilia B patients (68 males and 1 female with FIX = 0.08 iu/ml), 12 known carrier females and 1 normal female by a phenol-chloroform extraction method (9). These patients were attending the Haematology clinics at either the Queen Mary Hospital, Hong Kong; the Ramathibodi Hospital, Bangkok, Thailand or the Royal Free Hospital, London, UK. The genotype of the patients had been determined previously by direct genomic sequencing, but the results were blinded to the scientist performing the microarray analysis.

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To further validate the usefulness of the fabricated FIX array, DNA samples from 7 unrelated, new haemophilia B patients (mutations unknown) and 49 females (38 were relatives of known mutants and 12 were from new haemophilia B families) were sent from Thailand. The status of these samples were unknown to the Hong Kong group and to the laboratory personnel performing the microarray analysis.

All chemicals and solvents were of analytical grade purchased from Sigma-Aldrich (St. Louis, MO, USA) unless noted otherwise. Cyanine-labelled deoxycytosine triphosphate (Cy5-dCTP) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Pre-cleaned, non-derivatised glass microscope slides (25 x 75mm^2) were purchased from VWR Scientific (San Francisco, CA, USA) and LifterSlips were from Erie Scientific (Portsmouth, NH, USA).

Preparation of slides and array printing

The slides were prepared for printing and the oligonucleotide primers, approximately 56 basepair in length, with phosphorothioate incorporated at the 5' terminus and the allele-specific nucleotide at the 3' terminus were synthesized as described previously (8, 10). The oligonucleotide primers were designed to allow detection of known mutations in our initial 69 patients (primer sequences are available on request). The oligonucleotides were dissolved in 1 x spotting solution (Telechem, Sunnyvale, CA, USA) at two concentrations of 5 and 2 µM respectively and spotted onto the slide by contact printing with 'chipmaker' pins (Cartesian, Irvine, CA, USA) on the arrayer (Prosys gantry 5510, Cartesian). Each slide contains three sections of oligonucleotides for analysis of three different test samples. In addition, in each section, the oligonucleotides were spotted in triplicate. After spotting, the slides were left overnight in the humid chamber to allow the oligonucleotides to bind onto the glass surface.

Template preparation

Single-stranded template for allele-specific primer extension was generated by asymmetric amplification of the FIX gene in 7 fragments using either 50 ng (Exons 1, 4, 5 and 6) or 150 ng (Exons 2+3, 7 and 8) of genomic DNA in a 50 µl reaction volume containing 1 x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 3.5 mM $MgCl_2$, 40 nM forward primer, 200 nM reverse primer, 200 µM of each dNTP and 0.5 x BD Titanium Taq (BD Biosciences Clontech, Palo Alto, CA, USA). After an initial hotstart of 95°C x 30 sec, 50 cycles amplification of 95°C x 10 sec, 54°C x 20 sec and 68°C x 40 sec were performed, with a final extension time of 7 min.

All asymmetric-PCR (A-PCR) products were pooled, ethanol precipitated and resuspended in 24 µl water and column-purified (Auto-seq G50, Amersham Pharmacia Biotech, USA) to remove unincorporated dNTPS. Half of the A-PCR product was dried and resuspended in 5 µl of water for the analysis.

Allele-specific arrayed primer extension (AS-APEX)

To reduce non-specific binding, the arrayed slide was pre-incubated in 20 µl of 1 x PCR buffer under a LifterSlip (Erie Scientific, USA) in the hybridization cassette (Telechem, USA). The entire assembly was submerged in a 60°C water bath for 30 min. The slides were rinsed with water, spun dry at 110 g x 8 min, then overload with the AS-APEX reaction mix, containing A-PCR product, 0.25 µM of each dATP, dGTP, dTTP, Cy5-dCTP and 0.125 µM ddNTP, 0.75 x Titanium™ Taq Polymerase, 10 mM Tris HCl, 50 mM KCl, 1mM $MgCl_2$ and 4% DMSO in a total volume of 15 µl. The entire cassette assembly containing the array and LifterSlips were transferred to a 75°C water bath for 7 min, to activate the Titanium™ Taq Polymerase. The AS-APEX reaction was then allowed to proceed for a further 30 min in a 60°C water bath. The reaction was terminated by rinsing the slides three times with water at room temperature and in wash solution (0.5 x SSC and 0.03% sodium dodecyl sulphate) (1 x SSC = 0.3M NaCl, 0.03M sodium citrate) at 80°C for 2 min, then rinsed in 0.05 x SSC. After centrifugation (110 g x 8 min), the slides were scanned at an emission wavelength of 670 nm and 10 µm resolution for the detection of incorporated Cy5-dCTP (ScanArray 5000, GSI Lumonics, Boston, MA, USA).

Results

A FIX microarray allowing the detection of 69 mutations was fabricated. Initially, the array was tested on 82 samples from 69 unrelated Haemophilia B patients whose mutations were already known and 13 of their female family members. The results are shown in table 1. The mutations in 35 British, 22 Thai and 12 Chinese patients were correctly identified (accuracy = 100%). At the same time, 12 female carriers and 1 normal female were correctly identified.

The sample from a haemophiliac patient carrying a mutation nt 30929 A→T, gave a positive signal with the mutant oligonucleotide primer, but failed to extend (no signal) with both the normal primer for nt 30929 and nt 30933 (Fig. 1A). Similar results were observed for samples from patients carrying a mutation in close proximity to the next one, e.g., sample with nt 6 mutation failed to extend the normal primer at nt 6 and nt 8 (Fig. 1C).

Validation of the array using unknown samples

To test the usefulness of the array, a further 56 DNA samples were sent from Thailand. There were 49 females, 37 were relatives of patients with known FIX mutations. 14 obligate carriers were correctly identified and 14 out of 23 possible carriers were identified as carriers by array and confirmed by sequencing. The remaining nine possible carriers found to be normal by array analysis were subsequently confirmed as normal by genomic sequencing of their FIX gene. FIX:C level of the normal females were 51–90% whilst that of the carriers were 40–130% (Table 2A). 12 females were relatives of the 7 new haemophilia B patients whose mutations have not been characterised. FIX:C level had only been measured in four of these subjects (60–134%). Of these, four had their carrier status and mutation identified from the array analysis, and subsequently confirmed as correct by genomic sequencing. For seven females who were found to be normal on initial array analysis, sequencing of all eight exons of their FIX gene and the immediate 5' and 3' splice junctions did not reveal any mutations. The remaining case (N83) was identified as a carrier by sequencing, with a mutation at nt 6365 (G→T), which was not spotted on our array (vida infra).

Table 1: Results of array analysis for the detection of FIX mutations.

	Nucleotide Position	Mutation	FIX Patients			Females	
			British	Thai	Chinese	Carrier	Normal
1.	–26	G>T		1			
2.	–6	G>A	1				
3.	6	T>A			1	1	
4.	8	ΔT		1			
5.	57	ΔGA		1			
6.	122	G>C	1				
7.	6332	ΔT			1	1	
8.	6364	C>T	1	2			
9.	6365	G>A		3	2	2	1
10.	6410	G>A		1			
11.	6410	G>C			1	2	
12.	6443	G>A	1				
13.	6460	C>T		1			
14.	6461	G>A	1				
15.	6464/5	AA>TT	1				
16.	6676	A>G					
17.	6688	G>A	1				
18.	6704	T>G	1				
19.	10418	T>C		1			
20.	10430	G>A	2				
21.	10458	Δ4nts		1			
22.	10474	ΔC	1				
23.	10478	G>A	1				
24.	10479	G>T	1				
25.	10506	Δ4nts	1				
26.	17680	ΔA	1				
27.	17700	C>A	1				
28.	17810	A>G	1				
29.	20413	C>T	1				
30.	20519	G>A		1			
31.	20561	G>C		1			
32.	20562	G>A	1				
33.	30037	A>G		1			
34.	30038	G>A			1	1	
35.	30076	G>A	1				
36.	30150	G>A					
37.	30828	A>G	1				
38.	30863	C>T					
39.	30864	G>A		1			
40.	30873	T>A					
41.	30875	C>T			1	1	
42.	30929	A>T	1				
43.	30933	C>T	1				
44.	31008	C>T	1				
45.	31070	G>A		1			
46.	31118	C>T	1				
47.	31119	G>A	1				
48.	31122	C>A			1		
49.	31127	T>C	1				
50.	31127	T>G		1			
51.	31133	C>T		1			
52.	31136	T>C	1				
53.	31152	T>C			1	2	
54.	31163	A>G	1				
55.	31170	G>A	1				
56.	31171	T>G		1			
57.	31172	G>C			1		
58.	31196	G>A		1			
59.	31202	T>C	1				
60.	31212	A>T		1			
61.	31260	C>G			1	2	
62.	31260	C>T	1				
63.	31261	ΔT			1		
64.	31274	T>C					
65.	31289	G>A					
66.	31308	G>C	1				
67.	31311	T>C					
68.	31317	C>A	1				
69.	31328	C>T	1				
	TOTAL:		35	22	12	12	1

DNA samples of the seven new haemophilia B patients were analysed. The causative mutations of five patients were identified by array analysis (Table 2B) and confirmed by sequencing. In the remaining two patients, their DNA failed to extend the normal sequence at nt 20519 (G→A) (N67) and nt 6365 (G→A) (N84) respectively, this indicated that their FIX mutation lies at or immediately upstream to those regions. Genomic sequencing of their FIX gene revealed a new mutation at nt 20518 (C→T) and nt 6365 (G→T) respectively (Fig. 2). In the latter case, his female relative (N83) was also found by sequencing to be a carrier.

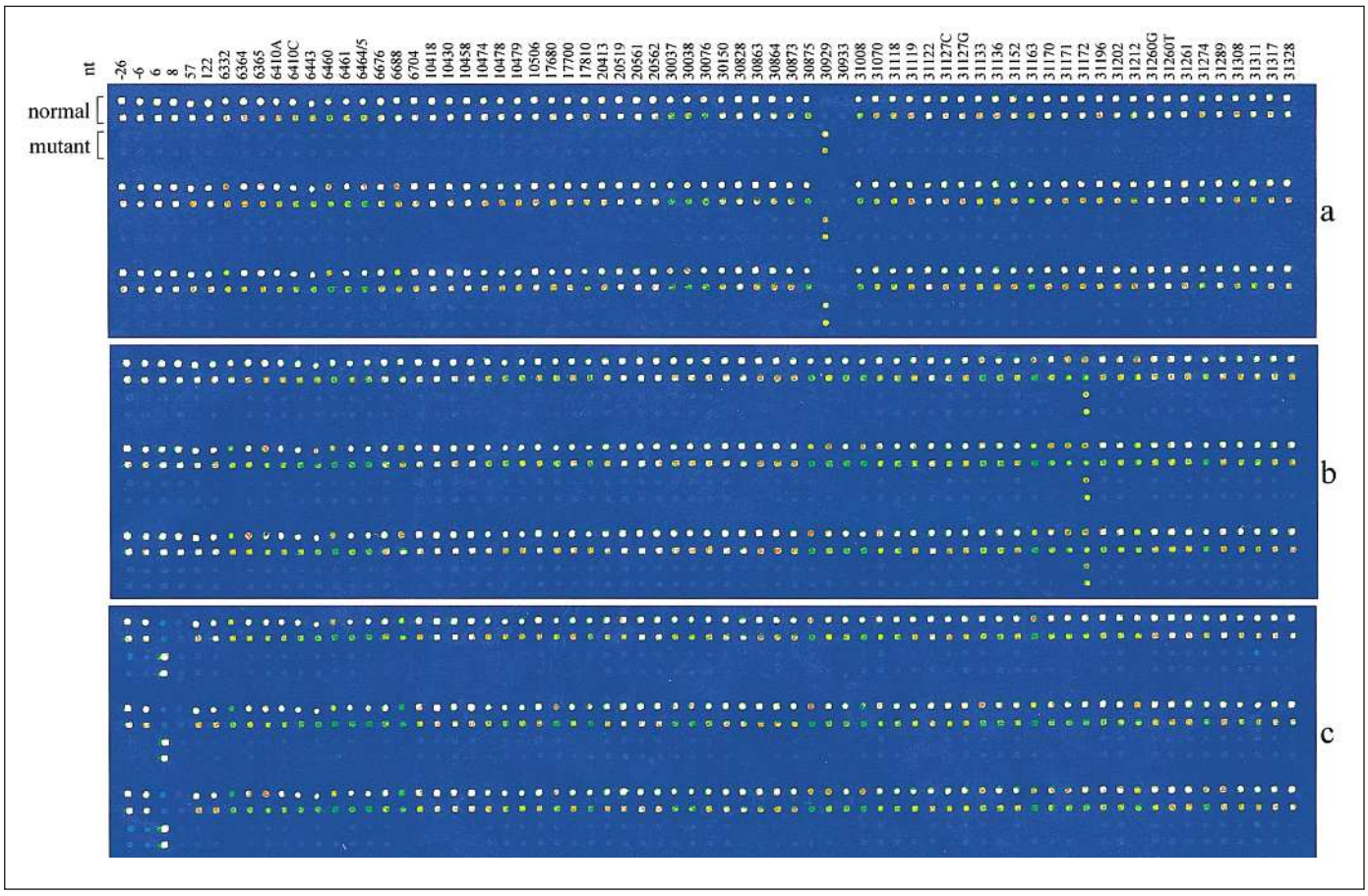

Figure 1: FIX array after hybridization of amplified genomic DNA from haemophilia B patients. The normal and mutant oligonucleotide primers were spotted in triplicate and at two concentrations, hence the positive signals after the AS-APEX reaction were of dual intensity. a) patient with mutation at nt 30929, b) A female patient who is heterozygous for the defect but has haemophilia due to skewed inactivation of her normal X chromosome and c) patient with defect at nt 6.

Discussion

In the current FIX array, we have spotted oligonucleotides for the detection of 69 mutations simultaneously. The majority of these mutations had been reported at least three times in the FIX database, while others were useful for detection of defects in Haemophilia B patients attending the Haematology clinic at our three centres. On a theoretical basis, any number (up to 8,000) of different oligonucleotide primers can be designed and spotted onto the array, if the entire surface of the slide (50 x 20 mm) is used for arraying and single test sample analysed on each slide. With the AS-APEX technology used in our array system, strand synthesis will only occur with a perfectly matched oligonucleotide and no stringent washing is needed to remove unstable complex as in allele-specific oligonucleotide (ASO) hybridization, which is the basis of the reverse dot blot (RDB) system. In comparison, RDB requires that the oligonucleotide probes be designed to allow hybridization and stringent washing for all the normal and mutant probes at the same temperature (11). Thus it would be impossible to design a RDB to detect the over 900 mutations presently identified for the FIX gene.

Since only 0.9 nl of oligonucleotide is arrayed onto each spot and the glass slide can be easily prepared for spotting, the cost of preparing our current FIX array in-house is approximately US$ 3.00. The array can then be used to analyse three test samples. It is possible to generate single stranded template for the AS-APEX reaction directly from genomic DNA by adjusting the asymmetric primers' concentration. In our hands, despite the number of amplification cycles needed, taking standard PCR precautions as in most routine laboratories that handle patient samples, no contamination or 'artificial' generation of mutations have been encountered. The cost of a sample analysis, including PCR reagents, AS-APEX reagent such as Cy5-dCTP and Titanium Taq Polymerase is US$ 8.10. This is much cheaper than the cost of automated sequencing of the whole gene (US$ 90 for unknown mutation detection, US$ 18 for subsequent carrier detection). Even if the known mutation alters an enzyme recognition site and PCR followed by direct restriction analysis is possible, the analysis using the FIX array would be less time-consuming, as the entire procedure of PCR and array analysis takes no more than six hours and also avoids the use of ethidium bromide for gel-staining which is hazardous to the laboratory staff.

Table 2A: Array analysis of female relatives of haemophilia B patients.

DNA No.	FIX:C	Carrier Status	Mutation of Haemophilia B patient		Array Analysis			Whole Gene Sequencing Result
					Carrier Detection	Genotype Identified		
N5	ND	O	10418	T→C	+	10418	T→C/T	
N7	48	O	31127	T→G	+	31127	T→G/T	
N18	110	O	30037	A→G	+	30037	A→G/A	
N20	80	O	30037	A→G	+	30037	A→G/A	
N22	104	O	8	ΔT	+	8	ΔT/T	
N29	56	O	6365	G→A	+	6365	G→A/G	
N30	70	O	6365	G→A	+	6365	G→A/G	
N31	132	O	6410	G→A	+	6410	G→A/G	
N38	20	O	6365	G→A	+	6365	G→A/G	
N54	110	O	6460	C→T	+	6460	C→T/C	
N61	ND	O	31212	A→T	+	31212	A→T/A	
N73	51	O	6365	G→A	+	6365	G→A/G	
N77	91	O	20561	G→C	+	20561	G→C/G	
N91	ND	O	31133	C→T	+	31133	C→T/C	
N10	ND	P	31127	T→G	-	31127	T	Normal
N13	90	P	31171	T→G	-	31171	T	Normal
N25	110	P	8	ΔT	-	8	T	Normal
N56	51	P	6365	G→A	-	6365	G	Normal
N64	ND	P	10458	ΔATGA	-	10458	ATGA	Normal
N70	ND	P	–26	G→T	-	–26	G	Normal
N81	85	P	20519	G→A	-	20519	G	Normal
N92	ND	P	31133	C→T	-	31133	C	Normal
N94	ND	P	6365	G→A	-	6365	G	Normal
N1	130	P	30864	G→A	+	30864	G→A/G	
N9	52	P	31127	T→G	+	31127	T→G/T	
N12	40	P	31171	T→G	+	31171	T→G/T	
N16	72	P	57/58	ΔGA	+	57/58	ΔGA/GA	
N34	25	P	6410	G→A	+	6410	G→A/G	
N44	72	P	57/58	ΔGA	+	57/58	ΔGA/GA	
N58	127	P	31196	G→A	+	31196	G→A/G	
N59	75	P	31196	G→A	+	31196	G→A/G	
N72	ND	P	31070	G→A	+	31070	G→A/G	
N75	69	P	6365	G→A	+	6365	G→A/G	
N80	81	P	20519	G→A	+	20519	G→A/G	
N95	42	P	6365	G→A	+	6365	G→A/G	
N96	ND	P	6364	C→T	+	6364	C→T/C	
N100	ND	P	20519	G→A	+	20519	G→A/G	
N105	ND	P	Unknown		+	6365	G→A/G	6365 G→A/G
N107	ND	P	Unknown		+	6365	G→A/G	6365 G→A/G

Table 2A: Continued

DNA No.	FIX:C	Carrier Status	Mutation of Haemophilia B patient		Array Analysis			Whole Gene Sequencing Result
					Carrier Detection	Genotype Identified		
N116	60	P	Unknown		+	6460	C→T/C	6460 C→T/C
N120	ND	P	Unknown		+	31133	C→T/C	31133 C→T/C
N49	ND	P	Unknown		-			Normal
N50	108	P	Unknown		-			Normal
N106	ND	P	Unknown		-			Normal
N110	ND	P	Unknown		-			Normal
N112	ND	P	Unknown		-			Normal
N117	134	P	Unknown		-			Normal
N119	73	P	Unknown		-			Normal
N83	ND	P	Unknown		-			6365 G→T/G

ND = Not done; O = Obligate; P = Possible; + Positive (carrier female); - Negative (normal female).

Table 2B: Mutation detection for 7 new haemophilia B patients.

DNA No.	Array	Sequencing	Mutation Identified	
N17	+	NR	30037	A→G
N67	+ Mutation around nt 20519	+	20518	C→T
N84	+ Mutation around nt 6365	+	6365	G→T
N104	+	NR	6365	G→A
N113	+	NR	31133	C→T
N115	+	NR	6460	C→T
N118	+	NR	20519	G→A

NR = Not required; + = Positive result..

Previous experience has shown that the spotted arrays are stable, can be stored at room temperature and are usable for at least six months. Thus it would be possible to have the array prepared in a single place and shipped to other laboratories for use, making it unnecessary for each laboratory to make their own array. The only requirement then would be a thermocycler and a laser scanner for detection of emission at a wavelength of 670 nm. This FIX array should be particularly useful for laboratories in some developing countries who may not wish to go to the expense of establishing high-throughout sequencing. The technique is relatively simple, includes generation of single-stranded template directly by PCR, performing the AS-APEX reaction on the array, single washing step and laser scanning. It would take less than a single working day to perform and is relatively cheap at around US$ 10 for a single sample analysis.

We have tested this FIX array initially on samples from 69 unrelated haemophilia B patients from England, Thailand and Hong Kong (whose mutations are known and arrayed on the slide) and some of their female family members (Table 1). It showed 100% accuracy. In AS-APEX technology, the allele-specific nucleotide is at the 3' terminus, resulting in extension and formation of a coloured (Cy5-labelled) extended product with a perfectly matched oligonucleotide primer. Thus the absence of coloured product (signal) with the normal oligonucleotide primer usually indicates 'homozygosity' or in case of a sample from the male patient, 'hemizygosity' or affected subject. One male with haemophilia had a mutation at nt 30929 A→T, but did not extend both its own normal oligonucleotide sequence as well as the normal sequence at nt 30933. The latter was most likely due to the formation of a 'bubble', where the mismatch occurred four nucleotides upstream, resulting in an unstable annealment between primer and template and therefore, no extension (Fig. 1A). This also explains the absence of extension in another patient with nt 6 mutation, for the normal sequence at nt 8 that lies two nucleotides downstream from the mutation under analysis (Fig. 1C).

To validate the usefulness of the microarray, we analysed a further 56 samples from patients and family members from Thailand. As expected, all 37 females from families with known mutation had their carrier status correctly determined by the array. Furthermore, in the seven new haemophilia B families, five had mutations detectable by the array while in the remaining two, their mutation was localized to the vicinity of a specific spot on the array. As discussed previously, this was due to the formation of an unstable complex between the 'mutant' template and the normal oligonucleotide primer a few nucleotide downstream, thus resulting in the absence of extension. Indeed, this was demonstrated in a sample from a new Thai Haemophilia B patient (N67) (Fig. 2). His DNA did not give any extension product with either the normal or mutant primers at nt 20519. Whilst this indicated that he did not carry the nt 20519 G→A defect, it meant that the mutation could be in the vicinity. Genomic sequencing of exon f of his FIX gene confirmed a mutation at nt 20518 C→T.

Thus it would be possible to use this array system as an initial screening technique for new Haemophilia B patients, to identify the causative mutation or to localize the region of the mutation along the gene. If undetected on the array, then conventional

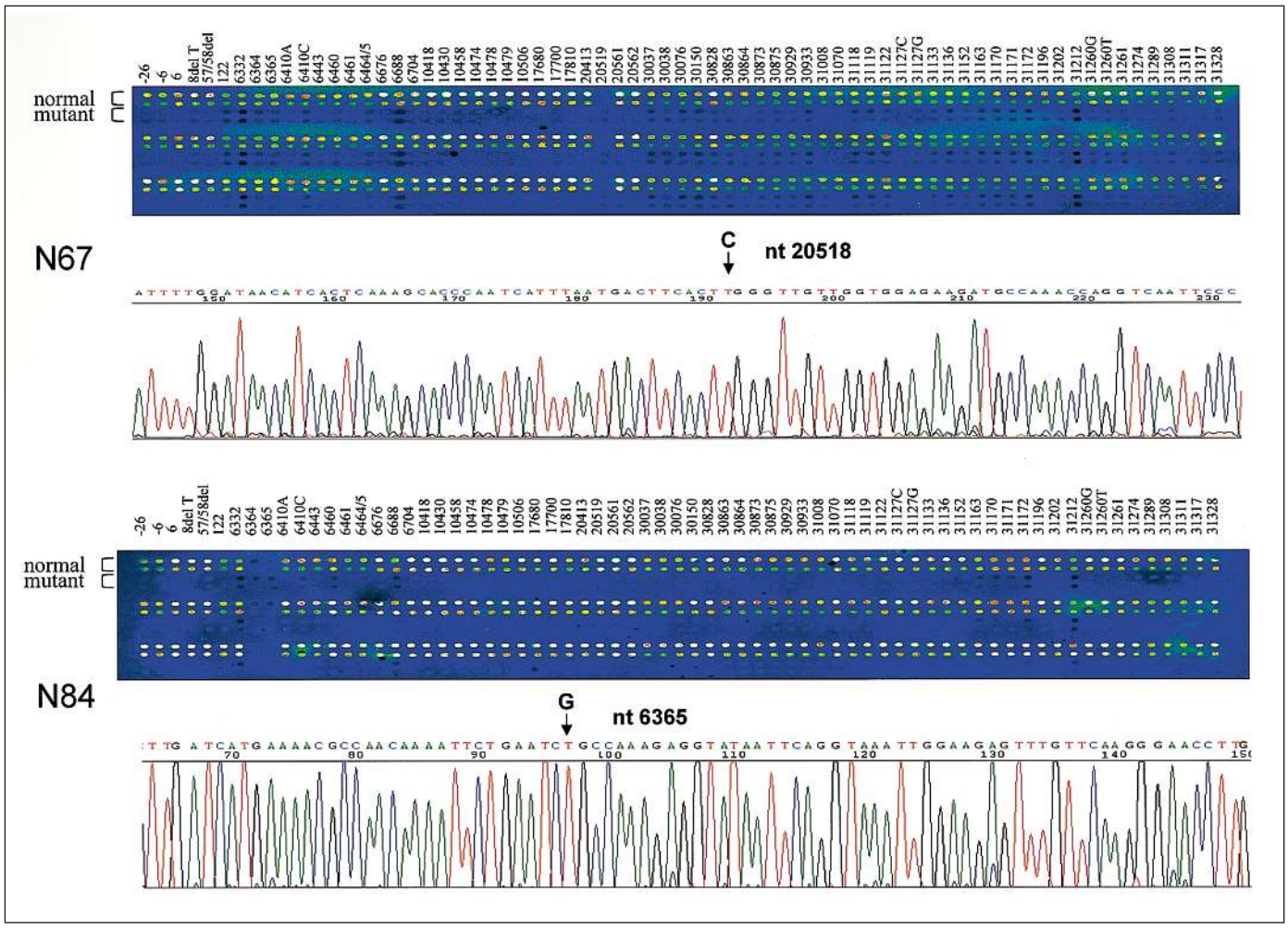

Figure 2: Location of two unknown Haemophilia B mutations by FIX array and their characterisation by genomic sequencing. FIX array analysis (panels 1 and 3) of amplified genomic DNA from two haemophilia B patients (N67 and N84), showing no extension product for both normal and mutant probes at nt 20519 and nt 6364 – 6365 respectively. The sequence profile for those respective region of the FIX gene of the two patients are shown underneath (panels 2 and 4). The normal nucleotide at the mutation site is shown in bold at the top of the sequence.

genomic sequencing of that particular exonic fragment will be performed. Based on this approach, the current array fabricated with 69 oligonucleotide primer sets will allow detection of 1537 out of the 2891 (53.1%) entries in the FIX database. Obviously it is possible to make a comprehensive array to detect all the entries (for over 900 FIX mutations) but it will be more expensive.

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