Mutation Detection by Fluorescent Chemical Cleavage: Application to Hemophilia B

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Chemical mismatch detection when combined with PCR or RT-PCR amplification represents one of the most efficient mutation screening methods. This procedure has been used for the analysis of both large populations of mutants and large and complex genes because it detects and locates virtually all sequence variations in segments of DNA of up to 1.7 kb cleaving at, and/or adjacent to, mismatches in heteroduplexes formed by target and probe DNA. By using fluorescent tags and an automatic gel scanning system, the efficiency of the method has been increased fourfold with gains also in the safety and quality control of probes and in the flexibility of the procedure. Using 12 hemophilia B patients, 8 with known mutations and 4 chosen at random, we show how the chemical mismatch cleavage of up to four DNA segments can be multiplexed and examined in a single gel lane and how the increase in efficiency thus obtained can be used either totally to maximize the DNA screened per lane or partly to locate the mutation unequivocally so that only one sequencing reaction is needed to characterize it fully.

The need to analyze mutations rapidly in increasingly larger populations and more complex genes demands continual improvement in efficiency of the methods that are available. Chemical mismatch detection reveals any sequence change in segments of up to 1.7 kb, and in contrast to the other two methods most used (denaturing gradient gel electrophoresis and single-strand conformational polymorphism), it locates the mutation within the segment analyzed.⁽¹⁻⁶⁾ These features have made it extremely useful in the investigation of very large and complex genes, especially when used to analyze the sequence of an mRNA amplified by reverse transcriptase-PCR (RT-PCR).^(3,4)

Chemical mismatch requires the hybridization of test and ³²P-end-labeled (probe) PCR products and treatment of the resulting heteroduplexes with osmium tetroxide and hydroxylamine. These chemicals modify mismatched T and C residues, respectively,⁽¹⁾ and, also sometimes matched T and C residues if they are adjacent to unpaired or mismatched bases. DNA is specifically cleaved at the modified bases by piperidine in denaturing conditions, and the products are resolved on a denaturing polyacrylamide gel. The presence of a unique band smaller than the full-length product reveals a mutation, and the size of the band indicates the distance of the mutation from one end of the molecule.

The ability to label DNA using four different fluorescent tags is a major advance that allows multiple PCR and mismatch reactions to be combined both during manipulation and gel analysis without loss of information. Thus, it al-

lows a fourfold increase in throughput; it can be used to provide more precise positional information, especially in the analysis of long PCR products, and it permits long-term storage of large amounts of stable probes. This approach is applicable to the study of mutations in any gene using chemical mismatch of either amplified genomic DNA or transcripts. We describe the application of fluorescent detection techniques to the study of mutations in hemophilia B.

MATERIALS AND METHODS

Amplification of Target and Probe DNA

DNA was extracted from peripheral blood cells by standard procedures.⁽⁷⁾ Fluorescent primers for the amplification of factor IX exons were purchased from Oswel. They were first synthesized with Aminolink 2 (ABI) at the 5' end and then derivatized with one of three fluorescent dyes (JOE, TAMRA, or ROX), followed by HPLC purification.⁽⁸⁾ The other dye-primer was synthesized directly using FLAM-phosphoramidite followed by HPLC purification.⁽⁹⁾ The following primers were synthesized to amplify, respectively, exons a, b and c, d, e, f, g, h and their flanking sequences: TAMRA-5a and TAMRA-3a; FAM-5b2 and FAM-3c; ROX-5d and ROX-3d; TAMRA-5e and TAMRA-3e; JOE-5f and JOE-3f2; FAM-5g2 and FAM-3g; JOE-5h2 and ROX-3h2. The sequence of the primers is as reported previously.⁽¹⁰⁾ Amplification of factor IX exons was performed as described previously.⁽¹⁰⁾ Probe DNA was amplified from normal genomic DNA using the fluorescently labeled primers in 100-μl reac-

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tions. Products were separated by electrophoresis, purified using GeneClean II (Bio 101), and eluted in a final volume of 20 μ l.

Fluorescent Mismatch Detection

The chemical cleavage procedure was performed as described previously,⁽²⁾ but fluorescent detection required a 5 $\times$ increase in probe and 8 $\times$ increase in target DNA. Multiplexed mismatch reactions consisted of 50 ng of each of three or four probes plus 400 ng of each corresponding target PCR product. These were denatured in 3 M NaCl, 0.3 M Tris-HCl (pH 8.0) for 5 min, then hybridized at 65°C overnight. The hybrids were ethanol precipitated and resuspended in 14 μ l of 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA. The hybrids were then treated with hydroxylamine (final concentration, 2.3 M) and osmium tetroxide (final concentration, 0.025%) and cleaved in 1 M piperidine exactly as described.⁽²⁾ After ethanol precipitation, the pellets were resuspended in 5 μ l of formamide, denatured for 5 min, electrophoresed through a standard 0.4-mm-thick, 24 cm 6% denaturing polyacrylamide gel run on a 373A DNA Sequencer (ABI) for ~14 hr, and analyzed using the 672 Genescan

software (ABI). Standard DNA molecular weight markers (Genescan 2500 ROX, ABI) were loaded in separate lanes.

Direct sequencing of PCR products was performed as described previously.⁽¹⁰⁾

RESULTS AND DISCUSSION

The eight exons of the factor IX gene and their flanking sequences were amplified in seven sections from normal genomic DNA using fluorescently labeled primers. These PCR products (the probes) were gel purified, stored at -20°C, and used in place of radioactively labeled probes in the chemical mismatch procedure.⁽²⁾ The corresponding sections from hemophilia B patients (the targets) were amplified using unlabeled primers.⁽¹⁰⁾ Multiplex 1 comprises three segments that contain exon a (labeled with TAMRA, yellow), exons b and c (labeled with FAM, blue), and exon h (labeled with JOE, green, at the 5' end and ROX, red, at the 3' end). The computer image of the gel (Fig. 1a) shows the full-size probe bands at 725 bp for exon h (where the green label dominates and hides the red), 653 bp for exon a, and 557 for exons b and c. Patients UK36, UK130, UK140, and UK53 were used to test mul-

tiplex 1, and mismatch bands are shown in Figure 1a. UK36 has a 10-bp deletion in exon a and so yields yellow cleavage bands with both chemicals and from both heteroduplexes at 590 nucleotides and 53 nucleotides (lanes 1,5). UK130 has a C $\rightarrow$ T transition at nucleotide 6364 that is 155 nucleotides from the 5' end of the PCR product. A blue mismatch band of 155 nucleotides can be seen in lane 2 resulting from the hydroxylamine reaction (lane 2). Exon h was labeled with different dyes at the 5' end of its two strands; this leads to unequivocal information on the location of the mutation, and in a large segment it reduces the sequencing required to characterize fully the gene defect. To test both dyes, multiplex 1 was hybridized with two patients (UK53 and UK140) carrying mutations such that one would lead to cleavage of the sense and the other of the antisense strand of the probe. Thus, patient UK53 has a G $\rightarrow$ A transition at nucleotide 31342 resulting in a C:T mismatch that is modified by hydroxylamine and cleaved to give a red (3') band of 99 nucleotides (lane 3). Because this is a red band, the mutation must lie 137 nucleotides from the red (3') end. Patient UK140 has a 1-bp deletion at nucleotide 30857 that results in a mismatch

FIGURE 1 Computer-generated gel display of fluorescent chemical cleavage analysis of hemophilia B genes. (a) Analysis of multiplex 1 (segment a, bc, and h) from patients UK36 (lanes 1,5), UK130 (lanes 2,6), UK53 (lanes 3,7), and UK140 (lanes 4,8). (b) Analysis of multiplex 2 (segments d, e, g, f) from patients UK28 (lanes 1,5), UK179 (lanes 2,6), UK117 (lanes 3,7), and UK27 (lanes 4,8). (Ha, Os) Hydroxylamine, osmium tetroxide treated, respectively. Size of segments and cleavage bands are shown (arrows). (c) Analysis of segment d of patient UK176 labeled in both probe and target strands.

band of 137 nucleotides with both hydroxylamine and osmium tetroxide (lane 4,8).

Multiplex 2, similarly tested on four patients (UK28, UK179, UK117, UK27), shows the full-length probes at 441, 298, 287, and 255 nucleotides representing exons f (green), e (yellow), g (blue), and d (red), respectively, and mismatch bands (Fig. 1b). A yellow mismatch band of 201 nucleotides from the hydroxylamine reaction can be seen in lane 1. This results from a C → A transversion in exon e in patient UK28 at nucleotide 17761. Patient UK179 has a G → T transversion at nucleotide 20548 (exon f) that gives the expected green mismatch band with hydroxylamine at 149 nucleotides (lane 2) but also a band at the same position with osmium tetroxide (lane 6). This is presumably the result of the T:A pair at position 20547 being destabilized by the adjacent mismatched base pair and therefore modified by osmium. Patient UK117 has a G → C transversion at nucleotide 30070 (exon g), and the expected 129-nucleotides blue mismatch band resulting from this mutation can be seen in lane 3. Finally, the test patient for exon d (patient UK27) has a G → A transition at nucleotide 10430 that gives rise to the expected red mismatch band at 126 nucleotides (lane 4).

The fluorescent mismatch detection procedure not only has detected all known mutations tested but has also identified four new ones (Table 1). The latter are all single-base-pair substitutions (G → T, G → A, A → G, G → C) affecting, respectively, exon b in multi-

plex 1 and exons d, e, and f in multiplex 2. These patients were simultaneously analyzed by the standard radioactive mismatch detection procedure, and the same results were obtained.

By combining up to four differently labeled PCR fragments in one tube and performing the mismatch reaction on this multiplex, four times the current maximum of DNA sequence can be screened in any one experiment, that is, ~7 kb per track per chemical (assuming 4 × 1.7-kb segments are tested per track). This defines two alternative positions for the mutation within one amplified segment because the identity of the cleaved strand is unknown.² Alternatively, in regions such as exon h of factor IX, which are large and a frequent site of mutation, the two ends of the PCR product can be labeled with different fluorescent tags so that the gel analysis indicates which strand of the probe has been cleaved and thus defines the unique location for each mutation. This allows the use of only one sequencing reaction to complete analysis of the mutation even in long DNA segments.

The only mismatches resistant to chemical modification and cleavage are G:T mismatches where the T is flanked on the 5' side by a G.⁽¹²⁾ Such mismatches can be detected through the C:A mismatch in the alternative heteroduplex. This, however, requires labeling of the patient DNA so that the mismatched C is in a labeled strand. By labeling both target and normal DNA and hybridizing them in equimolar amounts (100 ng), one can ensure the detection of these

mutations.⁽¹²⁾ The principle of using labeled target PCR products is illustrated using patient UK176. Exon d of the patient was amplified using red fluorescent primers and hybridized with the exon d probe (also labeled with the red dye). Figure 1c shows a band with both chemicals because one strand of both heteroduplexes is cleaved. If the G:T mismatch in patient UK176 had been resistant to cleavage, only the band in the hydroxylamine reaction would be seen because only the heteroduplex containing the C:A mismatch would have been cleaved. Only one mismatch band would be seen also in patients with A → T or G → C transversions, as in this case both cleavable strands would be in the same heteroduplex that will contain either a T:T or C:C mismatch.

As shown here, the use of fluorescent tags increases the throughput of the mismatch detection method in proportion to the number of colors detectable. According to experimental requirements, this gain in efficiency can be used entirely to increase the amount of DNA sequence screened per track or in part to locate the mutations unequivocally by marking the strands of the probe with different fluorescent tags. In addition, fluorescent labeling allows preparation of large quantities of the probe DNA, which can then be carefully quality controlled and stored. Finally, the automated gel system with its real-time scanning device allows greater resolution of large DNA fragments (1–2 kb) because the DNA is detected when it runs past a window three quarters of the way down the gel.

TABLE 1 Mutations Characterized

Patient no.	Color	Chemical	Band size	Exon	Nucleotide change type	Position
*UK36	yellow	Ha, Os	590, 53		Δ10	111–120
*UK130	blue	Ha	155	bc	C → T	6364
*UK53	red	Ha	99	h	G → A	31342
*UK140	green	Ha, Os	137	h	Δ1	30857
*UK28	yellow	Ha	201	e	C → A	17761
*UK179	green	Ha, Os	149	f	G → T	20548
*UK117	blue	Ha	129	g	G → C	30070
*UK27	red	Ha	126	d	G → A	10430
+UK111	blue	Ha	293	bc	1bpIns	6471
UK176	red	Ha	119	d	G → A	10437
+UK57	yellow	Os	260	e	A → G	17810
+UK131	green	Ha, Os	175	f	G → C	20524

(*) Mutations previously characterized;⁽¹¹⁾ (+) gel not shown. (Ha) Hydroxylamine; (Os) osmium tetroxide. (Δ) Deletion.

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