Denaturation Fingerprinting: Two Related Mutation Detection Methods Especially Advantageous for High G+C Regions

ABSTRACT

Two versions of denaturation fingerprinting (dnF_{2R} and dnF_{1R}) are described for detecting mutations. DnF_{2R} is a sensitive screening method in which fingerprints are generated by performing denaturing gel electrophoresis on bidirectional “cycle-sequencing” reactions with each of two dideoxy terminators, e.g., ddATP and ddCTP. When the fingerprints generated by ddATP and ddCTP are combined, all sequence changes are expected to result in one extra and one absent segment. DnF_{2R} was performed on 246- and 318-bp segments of the human factor IX gene, and the products were electrophoresed through a 6% Long Ranger gel with 7 M urea. All 32 single-base mutations were detected in hemizygous males with hemophilia B. DnF_{2R} has been applied to detect a total of seven heterozygous sequence changes in large-scale screening and was found to be especially suitable for high G+C regions.

INTRODUCTION

Mutation scanning methods are important for elucidating the genetic basis of human disease. Single-stranded conformation polymorphism (SSCP) is the most widely used mutation scanning method, but its sensitivity varies. Two hybrids between SSCP and Sanger dideoxy sequencing have been developed. These hybrid methods can detect the presence of virtually all mutations. Dideoxy fingerprinting (ddF) is a hybrid between SSCP and Sanger dideoxy sequencing. A Sanger reaction is performed with one ddATP and with one primer to produce a nested set of 5′ co-terminal DNA segments, then the segments are denatured and electrophoresed through a non-denaturing gel. Mutations can be detected by an alteration in the mobility of at least one of the multiple termination segments that contain the mutation (informative SSCP component). In addition, 6 out of 12 types of possible single-base substitutions result in a gain and/or loss of a dideoxy termination segment at the mutation site (informative dideoxy component). In manual gels, ddF can detect virtually all mutations in a 300-bp region of DNA. Bidirectional ddF (Bi-ddF) is a modification of ddF in which a cycle sequencing is performed with opposite primers to simultaneously scan for mutations in both directions. Bi-ddF has two important advantages over ddF: (i) the dideoxy component can detect 10 out of 12 types of possible single-base substitutions, and (ii) the SSCP component is on the average more informative because alterations of mobility can be detected in either the downstream or upstream direction. As a result, Bi-ddF can screen 600-bp segments with virtually 100% sensitivity. However, when these methods are adapted for high G+C regions, smearing of bands sometimes lowers the resolution.

Herein, we describe denaturation fingerprinting (dnF), an alternative to Bi-ddF without the problem of smearing in regions of high G+C. DnF was validated with exon H of the factor IX gene (40% G+C) on the X-chromosome and then applied for screening in the tumor necrosis factor α (TNFα) gene (>60% G+C) on an autosome.
MATERIALS AND METHODS

Oligonucleotides

All the oligonucleotides are specific for the human factor IX gene. The abbreviated informative names (9,11) are listed below:

- A: I7(30646)-34D; B: E8(31645)-31U; C: E8(30880)-21D; D: E8(31125)-16U; E: E8(31112)-16D; and F: E8(31429)-18U.

As an example of the nomenclature, I7(30646)-34D is an oligonucleotide in which the 5' end begins in intron 7 at bp 30646 [numbering as described in Yoshitake et al. (13)]. The length of the oligonucleotide is 34 bases, and the orientation is in “downstream” (D), i.e., in the direction of transcription. The precise sizes and locations of the amplified segments and the dideoxy termination reactions can be obtained from the informative names.

Mutations

For the analysis, the genomic DNA with the following mutations in the human factor IX gene were analyzed from patients with hemophilia B. The sample numbers and associated mutations correspond to the lane numbers for Figure 2 with primers C and D. 1: A30918G; 2: G31175A; 3: A31227G; 4: T31253G; 5: G31203T; 6: G31211T; 7: T31253G; 8: G31218A; 9: T31274A; 10: G31289A; 11: G31091T; 12: G31047A; 13: G31077A; 14: G31096A; 15: A31166A; 16: G31187T; 17: A31301G; 18: T31311C; 19: C31317A; 20: C31340C; and 16: C31356A.

PCR and Taq Cycling Sequencing

A 1-kb region of the exon 8 was amplified with primers A and B (4). Taq cycle sequencing was performed according to Innis et al. (3) with γ-33P[ATP]-labeled primers (Amersham, Arlington Heights, IL, USA). Denaturation was at 95°C for 15 s, annealing was at 55°C for 30 s and elongation was at 72°C for 4 min for a total of 20 cycles for primers C and D to screen a 246-bp region for primers E and F to scan a 318-bp region. The sequencing mixture contained a total volume of 8 μL: 80 mM Tris-HCl, pH 9.0, 2.0 mM MgCl2, 20 mM (NH4)2SO4, 10 μM of each dNTP, 1 U of AmpliTaq® (Perkin-Elmer, Norwalk, CT, USA), 10 ng of amplified DNA and 0.05 μM each of cycle sequencing primers C or D or E and F. In dN2F2R, each sequencing reaction contains 400 μM ddATP or 200 μM ddCTP as terminator; while in dN1R, the sequencing reaction contained 200 μM ddATP and 6000 μM ROX-ddCTP or (ddCTP*; PE Applied Biosystems, Foster City, CA, USA) 100 μM of ddCTP and 2250 μM of fluorescence-labeled ddATP (ddATP*). After Taq cycle sequencing, 16 μL of stop/loading buffer (7 M urea, 50% formamide and 2 mM EDTA) were added to each tube. The extension efficiency with the two opposite primers is 5–10-fold greater than that with either single primer.

Denaturing Electrophoresis

Electrophoreses of 7 M urea and 6% Long Ranger™ gels (0.4-mm thickness; FMC BioProducts, Rockland, ME, USA) were performed with a TBE buffer (50 mM Tris-borate, 1 mM EDTA, pH 8.3) at 65 W constant power. After pre-electrophoresis for 30 min, 1.5 μL of samples were loaded for 2 h, and the temperature on the plate...
was kept at 45°C. The gel was dried and subjected to Kodak BioMax MR film for autoradiography (Scientific Imaging Systems [Eastman Kodak], New Haven, CT, USA).

### Analysis of Resolution

Absent termination segments or extra segments were scored by visual analysis for the presence of unequivocal migration and intensity changes in relation to normal controls. If segments were crowded or compressed, a clearly distinguishable increase in the “complexity” of the segments or a 50% increase in segment intensity was judged to have an extra segment; and a reduction of complexity or a 50% reduction in segment intensity was judged to have an absent segment. A normal control was loaded every three lanes, so that a mutant sample always was immediately adjacent to a normal sample.

### RESULTS

#### Principle of the Method

In denaturation fingerprinting, Sanger dideoxy termination reactions are performed with two dideoxy terminators. One of which is either ddA or ddT, and the other is either ddG or ddC (Figure 1). Termination reactions are performed simultaneously in the downstream and upstream directions. In ddF2, the two Sanger termination reactions are performed separately, electrophoresed separately and then analyzed. For example, if a bidirectional cycle-sequencing reaction is performed with ddATP, ten of the twelve possible types of mutations will produce an extra segment, an absent segment or both (Table 1). If the second bidirectional cycle-sequencing reaction with either ddCTP or ddGTP is performed, all twelve possible sequence changes will be associated with one absent and one extra segment when both fingerprints are examined (Figure 1). For example, with an A→G mutation, a segment is lost when ddATP is used in the downstream termination reaction, and a segment is gained when ddCTP is utilized in the upstream termination reaction. The observed sensitivity should parallel these theoretical values if shadow segments are not a major problem, and all termination segments occur at reasonable intensities. It is not possible to combine the ddATP and ddCTP in one reaction because certain mutations would result in an absent and added base at the same site (Table 1). However, if one of the dideoxy nucleotides was modified so that the mobility changed by at least 1 bp, all types of mutations can be detected in one reaction (ddF1).

#### DnF of Two Regions of the Factor IX Gene

A 1-kb region of the exon 8 was amplified from genomic DNA with primers A and B. Cycle sequencing termination reactions with ddATP and ddCTP were electrophoresed through denaturing gels (see Materials and Methods). Two regions within this segment were analyzed by either ddF2 or ddF1 in hemizygous males with hemophilia B. These samples also should reflect the situation when patients are homozygotes and when clones are analyzed. To localize the mutation, a pair of control reactions was performed with just the downstream or the upstream primer. All sixteen of the mutations in the samples were detected in both a 246-bp region (201 bp if the primer sequences are eliminated) and an adjacent 318-bp segment (284 bp if

### Table 2. Summary of Experiments

<table>
<thead>
<tr>
<th>Genea</th>
<th>Segment Sizeb (bp)</th>
<th>G+C Content (%)</th>
<th>Type of Experimentd</th>
<th>Zygosity</th>
<th>No. of Mutations</th>
<th>dnF2R Detection</th>
<th>dnF1R Detection</th>
</tr>
</thead>
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<tr>
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<td>44</td>
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<td>Hemizygote</td>
<td>16</td>
<td>16</td>
<td>16</td>
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<tr>
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<td>16</td>
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<tr>
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<td>44</td>
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<td>24 (48)</td>
<td>24</td>
<td>24</td>
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<tr>
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<td>10c</td>
<td>11c</td>
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<tr>
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<td>19 (30)</td>
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<td>52–62</td>
<td>Screening</td>
<td>Heterozygote</td>
<td>4</td>
<td></td>
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aFIX = Human factor IX gene; TNFα = Human tumor necrosis factor α gene in which four regions were screened in patient samples.
bThe screening regions are assigned between the 5’ ends of the downstream and upstream primers.
cIn the 248-bp segment of the factor IX gene, two and one heterozygous mutations were missed due to weakly terminated segments by ddF2 and ddF1, respectively.
dIn the 318-bp segment of the factor IX gene, five heterozygous mutations were missed due to weakly terminated segments and poor separation on the upper part of the gel by ddF2 and ddF1, respectively.

The gene was screened in four regions with a G+C content ranging from 57% to 62%.

Known = the person performing the analysis knew which samples had mutations and which samples did not; blinded = the analysis was performed without knowledge of which samples contained mutations; screening = the gene was analyzed in a prospective manner in search of mutations.
the primer sequences are eliminated) (see Table 2 and Figure 2).

Although every mutation is expected to result in one extra termination segment and one lost termination segment, in practice, one or another segment may not be visualized. The extra termination segment may migrate in the same position as a termination segment from the opposite direction. This should occur approximately 25% of the time. In some cases, this will be manifested by a clear difference in intensity of that segment relative to those in the neighboring lanes. However, the great variation in termination efficiency from base to base with Taq DNA polymerase implies that a clear-cut intensity differ-

Figure 1. Schematic of dnF. A genomic region is amplified with primers A and B by PCR. Taq cycle sequencing is performed with two opposite primers C and D followed by a denaturing electrophoresis. For dnF$_{2R}$ (left), two terminators of ddATP and ddCTP are used separately in two reactions and then are electrophoresed through two lanes. For dnF$_{1R}$ (right), ddATP and a modified ddCTP* are used simultaneously in one reaction followed by a denaturing electrophoresis in one lane. Dideoxy termination segments from downstream and upstream primers are identified by controls that visualize termination products from just one direction. In the example shown, the amplified region contains single-base mutations. Mutation one (M1) is an A→C mutation. For dnF$_{2R}$, a segment is lost with ddATP from downstream primer in the first lane, and a second segment is gained with ddCTP from the downstream primer in the second lane. For dnF$_{1R}$, the extra and the absent segments also can be detected in one lane because mobility is retarded by the modified dideoxy terminator. Mutation two (M2) is a G→A mutation. A segment is gained with ddATP from downstream primer, the other segment is lost with ddCTP from upstream primer, which can be easily detected from downstream and upstream directions by both dnF$_{2R}$ and dnF$_{1R}$. Symbols: → = downstream segment, ← = upstream segment and * = segments terminated by modified ddCTP. The extra and missing termination segments at mutation sites are marked by arrows.
ence will not always be seen. In addition, occasional termination segments are too weak to be visualized on a gel. Thus, extra segments that do not overlap a termination segment in the opposite direction may not be visualized. For dnF1R, overlapping segments are expected about 60% of the time [i.e., for a random sequence of 50% G+C, the probability of an extra segment produced by ddATP not overlapping a segment produced by ddATP with the opposite primer or ROX-ddCTP with either primer is \((3/4)^3 = 0.42\)]. An analysis of the extra and lost segments in the above experiments revealed the expected frequency of unequivocal extra segments and lost segments for dnF2R. For reasons that are unclear, the frequency was higher than expected for dnF1R (56% of 64 extra or absent segments). In dnF1R, the ten lost segments and three extra segments that could not be detected resulted from a combination of compression in the upper part of the gel and low intensity of particular Taq termination products (Figure 2, see “?”). In addition to the above, a few mutant-containing termination products were also detected by SSCP-type

![Figure 2. DnF2R and DnF1R from experiment 1.](image)

(A) DnF2R with ddATP and primers C and D. Lane D: Wild-type control with a radiolabeled downstream primer. Lane U: with a radiolabeled upstream primer. Lane C: with radiolabeled downstream and upstream primers. Lanes 1–16 are mutant samples and their types and positions are listed in Materials and Methods. → = an extra or a lost segment at mutation site. ? = false negative and * = shifted segment due to SSCP effect. (B) DnF1R from experiment 1. DnF1R with ddATP and fluorescence-labeled ddCTP* and primers C and D. Same as in Panel A.
mobility shifts, which presumably resulted from residual secondary structure (Figure 2, see “*”). In dF_{2R}, thirteen mobility shifts were seen with eight of the mutations, and, in dF_{1R}, thirteen mobility shifts were seen with eleven of the mutations.

**Blinded Analyses**

Three blinded analyses were performed (Table 2). For each analysis, a mixture of mutant and wild-type samples were encoded such that the individual performing the analysis (QL) had no knowledge of which sample had mutations or how many total mutations there were. In the first blinded analysis, 48 samples were analyzed within the 248-bp region. There were 24 mutations not previously analyzed, and all were detected. There were no false positives. Additional blinded analyses were performed with heterozygote samples. Of 18 samples, 12 contained mutations within the 248-bp region. Two of these mutations were missed with dF_{2R}, and one was missed with dF_{1R}. Within the 318-bp region, a blinded analysis with 19 heterozygous mutations among 30 samples revealed that five mutations were missed. There were no false positives.

The **TNFα Gene; Four Regions with High G+C**

DnF_{2R} was utilized to detect mutations in tumor necrosis factor α, a gene with high G+C content of 52%–62%, in which ddF and bi-ddF smeared when performed at room temperature in the absence of denaturing reagents. Seventy-eight patients with multiple sclerosis were screened. The promoter region and the four exons and their flanking splice junctions were analyzed (total 1944 bp of sequence), and four heterozygous sequence variants were found, including three single-base substitutions and a one-base insertion (Table 2). These variants are reported elsewhere (12).

**DISCUSSION**

Two related methods are described. In dF_{2R}, two fingerprints are generated by performing bidirectional cycle sequencing separately with two ddNTPs (ddATP and ddCTP in the present experiments) followed by standard denaturing gel electrophoresis. A substitution results in one extra and one lost segment. One fingerprint (dF_{1R}) is generated by simultaneously using a regular ddNTP (e.g., ddATP) and another chemically modified terminator (e.g., ROX-conjugated ddCTP) that retards the mobility of the termination products. ROX-conjugated ddCTP and FAM-conjugated ddATP retard the migration of DNA segments by two nucleotides (8), while incorporation of Biotin 11-dUTP, a commercially available analog of TTP, causes a one nucleotide mobility shift (6). Ideally, a modified nucleotide would retard mobility by one and a half or two and a half nucleotides, such that extra and absent segments due to the mutation are less likely to migrate identically to another segment (e.g., hexyethylene oxide).

**Advantages and Disadvantages of dF**

For mutations in hemizygous, homozygous and cloned templates, dF_{2R} or dF_{1R} can detect virtually all mutations (56 of 56 tested mutations) with about one-half (dF_{2R}) or one-quarter (dF_{1R}) of the work, respectively, of sequencing. The sensitivity of dF_{2R} and dF_{1R} for heterozygotes may improve dramatically if: (i) the urea concentration is reduced to encourage more mobility shifts due to residual secondary structure while keeping most segments tightly focused for optimal resolution; (ii) the use of deoxy terminators that more optimally retard mobility, (iii) the use of sequencers in which segments must migrate at a defined distance to the detector (e.g., the PE Applied Biosystems or Pharmacia Biotech fluorescent sequencers) and (iv) the use of thermo sequencers (Amersham) or TaqFS DNA polymerase (PE Applied Biosystems) will produce more even-intensity termination segments.

The above modifications should enable the analysis of substantially longer segments.

DnF involves technology that is essentially identical to sequencing. Thus, the expertize in sequencing that so
many laboratories have can be applied directly to these methods. DnF is particularly advantageous for regions of high G+C content, such as promoter region, because segments may smear when Bi-ddF is performed in the absence of urea. However, substantial evidence indicates that increasing the urea concentration to 0.5 M for 60% G+C region, 1.5 M for 70% G+C regions resolves the problem without reducing the sensitivity at those G+C contents. While Bi-ddF and dnf may involve the use of urea for high G+C segments, Bi-ddF utilizes the SSCP effect as the primary mechanism of detecting mutation, while dnf uses the dideoxy component as the primary mechanism of detecting mutations and the SSCP effect is the secondary mode of detecting mutations.

DnF1R involves about half the work of dnf2R, but the pattern contains about two times as many segments. In blind analyses, dnf1R was as sensitive as dnf2R, so it seems to be the preferred technique. However, dnf1R is less forgiving; a suboptimal gel is more likely to lead to false-negative results. In summary, dnf2R and dnf1R are simple and effective methods for mutation scanning.

REFERENCES

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