S₁ nuclease hybrid analysis of mitochondrial DNA amplified by long-distance PCR: rapid screening for small-scale rearrangements

Katrin Lundin, Ekkehard Wilichowski*, Bernd-Peter Ernst and Folker Hanefeld

Universitäts-Kinderklinik, Abteilung Pädiatrie/Neuropädiatrie, Robert-Koch-Strasse 40, D-37075 Göttingen, Germany

Received March 24, 1997; Revised and Accepted April 18, 1997

ABSTRACT

We report on a method suitable for screening large regions (>3 kb) of mtDNA for structural changes of <500 bp and their localization. Heteroduplexes consisting of a wild-type and a mutant strand are cleaved by S₁ nuclease when single-stranded loops are present due to deletions or duplications/insertions. This strategy was successfully applied to screen the muscle mtDNA of 20 patients with mitochondrial encephalomyopathies. In three of them, an altered cleavage pattern was observed caused by a homoplasmic 9 bp deletion as shown by subsequent mapping and sequencing studies.

An increasing number of mtDNA alterations (deletions, duplications, point mutations) have been reported to be associated with mitochondrial encephalomyopathies (1) and degenerative processes like Parkinson’s syndrome, Alzheimer’s disease and even aging (2,3). The majority of mtDNA rearrangements are large-scale deletions and duplications spanning 1–11 kb or more (4). Although they are almost invariably present as a mixture of mutant and normal DNA (heteroplasmy), detection is simple by means of multi-primer amplification (5) or long-distance polymerase chain reaction (PCR) (6).

Small-scale rearrangements of <500–1000 bp have also been described but far more infrequently. Most of them are localized within noncoding mtDNA regions being either maternally inherited DNA polymorphisms (homoplasmic) or spontaneous somatic mutations in aging and other degenerative disorders (7). The scarcity of pathogenic small-scale aberrations associated with mitochondrial disorders (8,9) may be the result of difficulties in detection. Particularly when present at low heteroplasmic levels, methods widely used, including single strand conformational polymorphism (SSCP) and direct sequencing, are too insensitive or time-consuming in view of the genome size (16.6 kb) (10).

We have developed a method suitable for rapid and easy screening of large DNA regions for structural aberrations of small sizes. The strategy is a combination of DNA amplification of several thousand bp (4–7 kb) by long-distance PCR (11), and the use of S₁ nuclease to degrade single-stranded DNA (12). Amplified mtDNA of a control and the patient under study are mixed in equal amounts and denatured. Reannealing of DNA results in 50% homoduplexes consisting either of pure wild-type (25%) or pure patient’s (25%) DNA strands, and in 50% heteroduplexes consisting of both a wild-type and a patient’s DNA strand. If a deletion is present in the patient’s DNA, the corresponding segment of the normal strand will form a single-stranded loop in the heteroduplexes. On the other hand, if an insertion or duplication is present, a single-stranded loop will be generated by the patient’s DNA strand. These loops are removed by S₁ nuclease which degrades the single-stranded DNA portion and introduces a nick on the opposite strand. The resulting cleavage products can be visualized easily by agarose gel electrophoresis and ethidium bromide staining.

To validate this method, a deletion of 144 bp was created by digestion of purified platelet mtDNA with NarI (restriction sites at nucleotides 14 860 and 15 004) and religation with T4 DNA ligase (Pharmacia, Sweden).

Long-PCR was carried out in a 50 µl volume with 250 ng DNA as template and 10 pmol of each primer, using the long expand PCR kit (Boehringer Mannheim). Cycle conditions after an initial step of 92 °C for 2 min were: 30 cycles consisting of 92 °C for 20 s, 56–62 °C for 30 s, and 68 °C for 4–5 min with an increment of 20 s during the last 20 cycles, followed by a terminating step at 68 °C for 6 min. PCR fragments were ethanol precipitated and dissolved in H₂O to give a concentration of 1 µg/µl.

For heteroduplex formation, 8 µg each of purified mutant (144 bp deletion, patient’s DNA) and wild-type PCR fragments were mixed (1:1) in 50 µl hybridization buffer (0.6 M NaCl, 6 mM Tris–HCl, 10 mM MgCl₂, pH 7.7) and denatured (95 °C, 10 min). After cooling to 42 °C in 3 min, DNA was allowed to reanneal at 42 °C for 1 h. After desalting (Microspin columns S-200, Pharmacia) hybrids were incubated for 30 min at 37 °C with 330 U S₁ nuclease (Pharmacia, Sweden) in a final volume of 20 µl. Reactions were stopped by EDTA and samples were analyzed by electrophoresis on a 1% agarose gel.

As shown in Figure 1A, the model deletion of 144 bp was successfully identified in a PCR fragment of 4515 bp, using the experimental procedure described above. The detection of cleavage products of 3150 and 1220 bp confirmed the presence of the structural abnormality ~1 kb apart from the end of amplified DNA. Densitometric quantitation indicated DNA cleavage of less than the expected 50%. Further experiments demonstrated that this phenomenon is related to the size of template DNA used since the
proportion of restricted heteroduplexes approaches 50% with the decrease of template size to <1.0 kb (data not shown).

Using this screening method, we analyzed muscle mtDNA from 20 patients with different types of mitochondrial encephalomyopathies who were previously shown to harbour neither large-scale rearrangements nor one of the common point mutations. S1 nuclease hybrid analysis of five overlapping PCR fragments (of 4.5–6.35 kb) covering the entire mitochondrial genome revealed an identical alteration of banding pattern in five overlapping PCR fragments (of 4.5–6.35 kb) covering the entire mitochondrial genome.

The homoplasmic nature of this deletion was proven by further experiments required for definite characterisation of the detected rearrangement. It should also be applicable to nuclear DNA to screen for small structural aberrations in genes responsible for Mendelian inherited disorders.

ACKNOWLEDGEMENTS
This study was supported by grants from the Deutsche Forschungsgemeinschaft (GRK 60/1, Wi 1290/1-1). E.W. is recipient of a Deutsche Forschungsgemeinschaft fellowship (Wi 1290/1-2).

REFERENCES