Simultaneous A8344G heteroplasmy and mitochondrial DNA copy number quantification in Myoclonus Epilepsy and Ragged-Red Fibers (MERRF) syndrome by a multiplex Molecular Beacon based real-time fluorescence PCR

Károly Szuhai, Jody M. van den Ouweland, Roeland W. Dirks, Marc Lemaître1, Jean-Christophe Truffert1, George M. Janssen, Hans J. Tanke, Elisabeth Holme2, J. Antonie Maassen and Anton K. Raap*

Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands, 1Eurogentec S.A., Department of Production, Parc Scientifique, B-4102-Seraing, Belgium and 2Department of Clinical Chemistry, Sahlgrenska University Hospital, S-413 45 Gothenburg, Sweden

Received October 13, 2000; Revised and Accepted December 7, 2000

ABSTRACT

The association of a particular mitochondrial DNA (mtDNA) mutation with different clinical phenotypes is a well-known feature of mitochondrial diseases. A simple genotype–phenotype correlation has not been found between mutation load and disease expression. Tissue and intercellular mosaicism as well as mtDNA copy number are thought to be responsible for the different clinical phenotypes. As disease expression of mitochondrial tRNA mutations is mostly in post-mitotic tissues, studies to elucidate disease mechanisms need to be performed on patient material. Heteroplasmy quantitation and copy number estimation using small patient biopsy samples has not been reported before, mainly due to technical restrictions. In order to resolve this problem, we have developed a robust assay that utilizes Molecular Beacons to accurately quantify heteroplasmy levels and determine mtDNA copy number in small samples carrying the A8344G tRNALys mutation. It provides the methodological basis to investigate the role of heteroplasmy and mtDNA copy number in determining the clinical phenotypes.

INTRODUCTION

In the past decade mitochondrial genome variation has been recognized as a contributor to human disease. More than 50 pathogenic mitochondrial DNA (mtDNA) mutations have been described so far. The majority of these mutations are single base changes that occur in tRNA genes (1). Maternal inheritance is a specific feature of mtDNA (2) and diseases associated with point mutations in mtDNA are involved in several, predominantly neuromuscular diseases. They are also thought to play a role in aging due to the accumulation of mtDNA damage as a consequence of a low efficiency repair system and absence of protective proteins such as histones (3).

The influence of many pathogenic mtDNA mutations on mitochondrial transcription, protein synthesis and oxidative phosphorylation has been described (4,5) and is still the subject of intensive study (6–8). A well-studied example is the A8344G tRNALys mutation that causes Myoclonus Epilepsy and Ragged-Red Fibers (MERRF) syndrome (9–11). This disorder represents an encephalomyopathy characterized by myoclonic and tonic-clonic seizures, ataxia, dementia, muscle weakness and mitochondrial abnormalities in skeletal muscle. The same A8344G mutation is found in multiple, symmetric lipomas as the only manifestation of the mutation in non-symptomatic relatives of MERRF patients (12).

The association of a particular mtDNA mutation with different clinical phenotypes is also seen for other mutations. For example, the A3243G tRNALeu mutation leads in some individuals to the mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episode syndrome (MELAS) (13), and in others to the maternally-inherited diabetes and deafness syndrome (MIDD) (14) or to progressive kidney disease (15). A characteristic feature of the A8344G tRNALys and other pathogenic mutations is that tissues harbor a mixture of mutant and wild-type mtDNA, a phenomenon referred as heteroplasmy. Different tissues of a patient often show different levels of heteroplasmy. This variability in heteroplasmy has been suggested to be fundamental to the diversity of clinical presentation of a particular mutant. Cellular mosaicism (i.e. heterogeneous distribution of heteroplasmy) may be an additional factor involved in the variable clinical expression (16–18).
Furthermore, the mitochondrial copy number may contribute to the clinical expression. Studies with cybrid cell lines clearly illustrated that, in addition to the level of heteroplasmy, the mtDNA copy number is a limiting factor in cell respiration and consequently influences the phenotype (19).

For the understanding of the patho-biochemistry of mitochondrial diseases it is therefore essential to have a method to quantify heteroplasmy levels in tissues, in small groups of cells or in single cells.

In this study we have developed an alternative, PCR-based assay for heteroplasmy quantification of the A8344G rRNA (26) mutation in tissue samples that uses two differently labeled (mutant and wild-type) Molecular Beacon probes for real-time fluorescence monitoring of PCR. In addition we show that by applying a Molecular Beacon-PCR for the β-globin gene, a mitochondrial genome quantitation relative to the nuclear genome copy number can be achieved. Molecular Beacons are well suited for this purpose because they are fluorogenic, permit multiplexing and have great discriminatory power for single base mismatches due to the stem–loop structure of the molecule (20).

**MATERIALS AND METHODS**

### Patient and cell line DNAs

The DNA samples derived from 10 carriers of the A8344G mutation with or without clinical manifestation of MERRF syndrome. From each individual one tissue sample was analyzed (lymphocytes, muscle biopsy or lipoma). DNA was extracted as described (21). The samples were selected to cover a wide range of mtDNA heteroplasmy with the A8344G mutation. The restriction fragment length polymorphism (RFLP)–PCR-based mutation analysis was done as described (22). Briefly, a 32P-labeled forward primer was added before the last PCR cycle. After restriction enzyme cleavage and electrophoresis the percentage of mutated to normal mtDNA was determined by a PhosphorImager as described (23). R1-C3 cells carrying the rRNA (26) mutation and R2-1A cells carrying the same mtDNA without the mutation (4) were used for assay development. Cell lines were cultured in Dulbecco’s modified Eagle’s medium containing 50 µg/ml uridine, supplemented with 10% fetal bovine serum. Total DNA was isolated from all cell lines using standard procedures (24). DNA samples from R2-1A and R1-C3 cell lines were mixed to simulate 5, 10, 25, 50, 75, 90 or 95% heteroplasmy at DNA concentrations of 2 ng/µl. To assess the sensitivity of PCR reactions, a series of DNA dilutions was prepared from R1-3C cell DNA (100 ng/µl down to 0.1 pg/µl); 1 µl was used in each PCR reaction.

### Molecular Beacon design

Molecular Beacon probes were designed following the guidelines described by Tyagi and Kramer (25) and in the related Web site (www.phri.nyu.edu/molecular_beacons). The intramolecular configuration of Molecular Beacons and the amplicon probing were modeled using the DNA MFold program (www.ibr.wustl.edu/~zuker/dna/form1.shtml) that uses thermodynamic parameters established by SantaLucia (26). Molecular Beacons for the mutant and wild-type rRNA (26) did not contain self-complementary sequences of the tRNA (26). Both Beacons were equipped with a 6 bp stem sequence. Melting temperatures of probe sequences were calculated using Primer3 (Web site, http://www.williamstone.com). Candidate PCR primers and Molecular Beacons were checked for inter- and intramolecular interactions with the same software. Molecular Beacons were synthesized using standard phosphoramidite chemistry and purified by high performance liquid chromatography (HPLC). Briefly, Molecular Beacons were synthesized automatically with the 3′-DABCYL quencher linked to the solid phase support (DABCYL-CPG, Glen Research). Oligonucleotide chain elongation was run via standard cyanoethyl-phosphoramidite chemistry and fluorophores were covalently linked to the 5′-end via Iodoacetamide (Molecular Probes) or phosphoramidite derivatives (Glen Research) following release from the solid support. Molecular Beacons were purified twice by reverse-phase HPLC with a Waters 600E equipped with a Waters 996 Photodiode Array Detector for simultaneous detection at three different wavelengths. Ion-molecular weights of purified Molecular Beacons were determined by mass spectrometry using a Dynamo Time-Of-Flight instrument. The temperature at which there is no contribution of mutant Molecular Beacon to the fluorescence from wild-type target and of wild-type Molecular Beacon to mutant target was determined as described (25).

### Primers and Molecular Beacon probes

Sequences of primers and Molecular Beacon probes are described in Table 1. β-Globin gene-specific primers (RS40/RS42) were described by Greer et al. (27).

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS40</td>
<td>5′-TTTTCCCCACTTTAGGCTG-3′</td>
</tr>
<tr>
<td>RS42</td>
<td>5′-CTACCTCAGTGCGCAAG-3′</td>
</tr>
<tr>
<td>47ex (8259–8281)</td>
<td>5′-TTACCCCTATGACCCCCCCTTAC-3′</td>
</tr>
<tr>
<td>51ex (8396–8371)</td>
<td>5′-TGGGCCATACGTTATTTAGTTGG-3′</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Molecular Beacons</th>
<th>WT-594 (8334–8353)</th>
<th>TET-5′-CCACCG GATTAAGAGAACCACCG CGCTGG-3′-DABCYL</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-593 (8334–8353)</td>
<td>FAM-5′-CCACCG GATTAAGAGAACCACCG CGCTGG-3′-DABCYL</td>
<td></td>
</tr>
<tr>
<td>BG-736</td>
<td>FAM-5′-CCACCG CTGTCCTCCTGATCTGTTAT CGCTGG-3′-DABCYL</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1. Sequences of amplification primers and Molecular Beacon probes**

### PCR conditions

Two-color Molecular Beacon PCR reactions were carried out in the presence of 1× PCR buffer (containing 15 mM Tris–HCl pH 8.0 and 50 mM KCl), 5 mM MgCl2, 200 µM of each deoxy-nucleotide-triphosphate (dATP, dCTP, dGTP, dTTP), 1 U of AmpliTag Gold DNA polymerase (all from Applied Biosystems), 250 nM of each primer, 200 nM of 6-carboxyfluorescein (FAM)-labeled mutant, 200 nM tetrahydrodichloro-6-carboxyfluorescein (TET)-labeled wild-type Molecular Beacons and 2 µM of each primer, 200 nM of 6-carboxyfluorescein (FAM)-labeled mutant, 200 nM tetrahydrodichloro-6-carboxyfluorescein (TET)-labeled wild-type Molecular Beacons and...
1 μl of sample DNA in 50 μl final volume. Amplification reactions were performed in an ABI Prism 7700 spectrofluorometric thermal cycler (Applied Biosystems) with the following cycle conditions: 95°C denaturation and enzyme activation step for 10 min followed by 40 cycles of 95°C denaturation for 15 s, 59°C annealing for 30 s and 72°C elongation for 30 s. For the β-globin gene amplifications identical PCR conditions were used. Fluorescence spectra were recorded during the annealing phase of each PCR cycle. The Sequence Detection System software (SDS v1.7) of the ABI-Prism 7700 was used to generate the FAM and TET fluorescence intensity values.

**Heteroplasmy quantification and threshold cycle determination**

Since Molecular Beacons are reporting hybridization events of single-stranded DNA molecules, the integrated fluorescence intensities of each Molecular Beacon are proportional to the amount of cognate amplicons. To quantify the contribution of the mutant and wild-type sequence variants to the mtDNA PCR amplicons, fluorescence intensities of the differently labeled Molecular Beacons were integrated during the exponential phase of the PCR. In our experience, this phase comprises seven or more cycles (Fig. 1A and B). Thus integration was done over cycles $n_{\text{max}} - 3$ to $n_{\text{max}} + 3$, where $n_{\text{max}}$ is the cycle number at which maximum amplification occurs. To objectively determine $n_{\text{max}}$, the first derivative of the amplification curve was taken. In cases where no amplification was obtained with one of the A8344G Molecular Beacons (homo-plasmy), the interval of integration was defined by the interval determined with the responding Molecular Beacon, (Fig. 1C, open versus filled symbols).

**Threshold cycle calculations**

The threshold cycle number (Ct) was calculated with SDS software v1.7 (Applied Biosystems) and an automatic setting of the baseline. The baseline value was the average fluorescence value of PCR cycles 3–15 plus 10 times its standard deviation (SD). These values were used for the relative copy number calculations by expressing Ct differences of the β-globin and mtDNA PCR.

**RESULTS**

**Quantification of heteroplasmy levels and sensitivity of PCR reactions**

For heteroplasmy quantification, independent of input mtDNA copy number, the fluorescence intensities of the wild-type and
mutant A8344G Molecular Beacons were integrated over seven cycles of the exponential phase of the PCR reaction. This approach was validated by analyzing DNA samples with known amounts of mutant and wild-type mtDNA (Fig. 1A and B). As can be seen in Figure 1D, there is a linear relationship between the measured level of heteroplasmy and the nominal input values. Only a small correction is necessary for determining heteroplasmy levels of test samples. An unequivocal detection of as little as 5% heteroplasmy was possible.

Subsequently, heteroplasmy levels were determined in a set of 10 DNA samples. Figure 2 shows Molecular Beacon PCR results for three of the 10 samples. As shown in Figure 3, a linear correlation exists between the heteroplasmy values determined with the Molecular Beacon PCR approach and by the classical RFLP method. Note that the Molecular Beacon-PCR data were determined from seven independent measurements (averaged SD = 4.22).

Also, the accuracy of heteroplasmy quantification using the ‘Ct approach’ was analyzed (28). For the ‘simulation’ experiment in Figure 1A, B and D, r² of 0.834 and 0.9943 was found for the Ct and integration approach, respectively. Also, for the patient samples, RFLP-PCR data were compared with the classical RFLP method. Note that the Molecular Beacon-PCR data were determined from seven independent measurements (averaged SD = 4.22).

To be able to relate mitochondrial to nuclear genome copy number and to assess whether sensitivity of the heteroplasmy quantitation assay is at the single cell level, DNA was diluted 10-fold stepwise, and diluted samples were subjected to mitochondrial A8344G as well as to β-globin Molecular Beacon PCR. The β-globin Molecular Beacon PCR allowed reliable detection of the nuclear gene from as little as a single-cell equivalent of DNA. As expected, the mitochondrial tRNA Lys gene is readily amplified from even smaller amounts. As can be seen in Figure 4 an inverse linear relationship was found between the Ct values and the logarithm of the input DNA. Moreover, the slopes for the mitochondrial tRNA L53 and the β-globin gene are similar, indicating that amplification efficiencies of these two different targets are identical. Consequently, Ct values can be used as a measure of the input copy number and Ct value differences used to quantify mtDNA copy number relative to the β-globin gene with the following equation: $R_c = 2^{\Delta C_{t_{\text{Ct}}}}$ (28), where $R_c$ is the calculated relative copy number and $\Delta C_{t}$ is the $C_{t_{\text{Ct}}} - C_{t_{\text{mtDNA}}}$. The results are shown in Table 2. There appear to be large differences in relative mitochondrial copy number between the different samples. In accord with previous findings (29,30) the mtDNA copy number of the three lymphocyte samples (439, 449, 1586) showed the lowest values. Muscle samples were found to have high mtDNA copy numbers, except for sample number 470, which derived from a two and a half-year-old child, whereas the other samples were from teenagers or adults. No correlation was found between copy number and heteroplasmy level (Fig. 5).
Table 2. Mitochondrial copy number relative to β-globin gene content as determined from the Ct differences

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>β-globin Ct</th>
<th>mtDNA Ct</th>
<th>ΔCt (^a)</th>
<th>R (^b)</th>
<th>Tissue type</th>
</tr>
</thead>
<tbody>
<tr>
<td>208</td>
<td>29.8</td>
<td>17.1</td>
<td>12.7</td>
<td>6650</td>
<td>Muscle</td>
</tr>
<tr>
<td>386</td>
<td>32.2</td>
<td>20.4</td>
<td>11.8</td>
<td>3570</td>
<td>Muscle</td>
</tr>
<tr>
<td>405</td>
<td>32.3</td>
<td>20.5</td>
<td>11.8</td>
<td>3570</td>
<td>Muscle</td>
</tr>
<tr>
<td>406</td>
<td>32.6</td>
<td>19.9</td>
<td>12.7</td>
<td>6650</td>
<td>Muscle</td>
</tr>
<tr>
<td>439</td>
<td>28.4</td>
<td>20.5</td>
<td>7.9</td>
<td>240</td>
<td>Lymphocyte</td>
</tr>
<tr>
<td>449</td>
<td>31.4</td>
<td>23.5</td>
<td>7.9</td>
<td>240</td>
<td>Lymphocyte</td>
</tr>
<tr>
<td>470</td>
<td>31.1</td>
<td>21.1</td>
<td>10.0</td>
<td>1020</td>
<td>Muscle</td>
</tr>
<tr>
<td>600</td>
<td>30.5</td>
<td>19.4</td>
<td>11.1</td>
<td>2190</td>
<td>Lipoma</td>
</tr>
<tr>
<td>960</td>
<td>32.5</td>
<td>21.5</td>
<td>11.0</td>
<td>2050</td>
<td>Muscle</td>
</tr>
<tr>
<td>1586</td>
<td>29.0</td>
<td>20.3</td>
<td>8.7</td>
<td>420</td>
<td>Lymphocyte</td>
</tr>
<tr>
<td><strong>MERRF cybrid</strong> (^c)</td>
<td>30.99 ± 0.43</td>
<td>20.8 ± 0.34</td>
<td>10.2</td>
<td>1180</td>
<td>143B TK</td>
</tr>
</tbody>
</table>

\(^a\)ΔCt = Ct_\text{globin} – Ct_{\text{mtDNA}}.
\(^b\)R \(_c\), relative mtDNA copy number.
\(^c\)All cybrid mixtures used for the calibration curve (n = 9) were included for this Ct analysis.

**DISCUSSION**

The variations in clinical phenotypes of mitochondrial diseases are thought to be the result of variations in heteroplasmy levels in different tissues. When the proportion of the wild-type mitochondrial genome decreases below a certain level, cells become dysfunctional, a phenomenon known as the threshold effect. However, it has not been clarified whether the proportion of mutated mtDNA in a tissue is the only major factor for the phenotypic expression of the mutation. Cellular mosaicism (i.e., heterogeneous distribution of heteroplasmy) may be an additional factor involved in the variable clinical expression patterns (16,18). Also, mitochondrial copy number may be an influence on phenotypic expression (19). Increased mitochondrial proliferation is indeed characteristic of heteroplasmy. Increased mtDNA copy number relative to the wild-type mtDNA genome in real-time under homogeneous conditions can cause drift in heteroplasmy. Inter- and intra-cellular variation in mtDNA copy number may be responsible for delayed clinical manifestations.

A recent mathematical simulation of heteroplasmy drift in postmitotic heterocellular cells illustrated how increased mtDNA replication can cause drift in heteroplasmy. Notably, mtDNA copy number in small groups of cells or single cells is essential for understanding mitochondrial disease mechanisms. The present methodologies are unable to address this problem. Bicolor in situ hybridization, similar to that described for Pearson’s syndrome with large-scale mtDNA deletion (17), would have the requisite spatial resolution but lacks the specificity and sensitivity for single base mismatch detection as needed for the majority of mtDNA mutations. In order to address the problem of mtDNA copy number changes caused by a heteroplasmy drift in patient samples an assay with low material demand is needed. The currently available assays for these are a Southern blot-based copy number determination and a PCR amplification followed by RFLP analysis. Southern blotting has a high material demand and both assays are tedious.

Here we developed a homogeneous PCR assay that uses Molecular Beacons in a duplex format to quantify point mutations in mtDNA. A relative low mtDNA copy number was found in muscle tissue of MERRF cybrid cell lines. Heteroplasmy levels, determined from the same samples are indicated.

**Figure 5.** Relative mtDNA copy numbers from various tissues derived from the set of 10 samples of the A8344G mutation carriers and the mixes of the MERRF cybrid cell lines. Heteroplasmy levels, determined from the same samples are indicated.
copy number is provided. Thus, in combination with microdissection, our approach provides the methodological basis for the study of heteroplasmy and mtDNA copy number in relation to clinical phenotypes.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Giuseppe Attardi for the cybrid cells, Dr Ronald van Soest for initial assistance with the ABI-PRISM 7700, Dr Fred Kramer, Dr Sanjay Tyagi and Salvatore Marras for advice on Molecular Beacons.

REFERENCES


