Quantification of the common deletion in human testicular mitochondrial DNA by competitive PCR assay using a chimaeric competitor

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The ‘common’ 4977 bp deletion in mitochondrial DNA (∆4977) is commonly used as an indicator of tissue deterioration in ageing and bioenergetic diseases. Deletion levels are normally measured by a serial dilution polymerase chain reaction (PCR) approach, where test reactions are compared with dilutions of control amplifications of DNA from a similar sized stable region of the mitochondrial genome. The end-point of this assay is the dilution that can just detect any PCR product; however, this is an inherently unstable measure. We constructed a chimaeric DNA construct that binds to both control and deletion primers with similar annealing properties. This was used in a competitive PCR assay to quantify ∆4977 in human testicular tissues that had been well-characterized using the serial dilution approach. We found the competitive assay to be highly replicable as it compares the PCR product of the construct with that of test DNA samples during the linear growth phase of the PCR reaction. Moreover, the serial dilution assay was shown to significantly overestimate the amounts of deleted mitochondrial DNA present. The assay promises to throw new light on the role of mitochondrial DNA deletions in tissue dysfunction and ageing, as such deletions can now be determined with high accuracy and repeatability and is much cheaper to apply than real-time fluorescent quantitative PCR.

Key words: common deletion/competitive PCR/mitochondrial DNA/polymerase chain reaction

Introduction

Mitochondria are semi-autonomous organelles dedicated to the production of energy by oxidative phosphorylation and generally (but not exclusively) transmitted through the female germline (Cummins, 1998). While the sperm tail and mid-piece normally enter the oocyte at fertilization (Ankel-Simons and Cummins, 1996), the paternal mitochondria do not normally survive beyond the morula stage. This is a species-specific proteolytic mechanism mediated by ubiquitination of the sperm mitochondria during spermatogenesis (Sutovsky et al., 2000). Normally the paternal mitochondria do not survive, except in unusual situations such as inter-specific hybrids (Kaneda et al., 1995) and artificial cytoplasmic constructs (Meirelles and Smith, 1997, 1998). The onset of intracytoplasmic sperm injection (ICSI) in human assisted reproduction has led to concerns that this might lead to iatrogenic induction of mitochondrial disease in offspring, through the injection of abnormal spermatozoa that might evade destruction (Cummins and Jequier, 1995; Houshmand et al., 1997). Alternatively, aberrant embryos may lack adequate proteolytic mechanisms for eliminating the sperm mitochondria (St John et al., 2000). We have shown that even normozoospermic men have significant levels of deletions in their semen, probably arising as a result of oxidative stress (Cummins et al., 1998). There is also concern that technologies using cytoplasmic transfer to ‘rescue’ poor oocytes or embryos might generate abnormal individuals through heteroplasmy (Barritt et al., 1999; Cummins, 2001b), and the poor outcomes currently seen with cloning technology may also be based on aberrant nuclear–cytoplasmic cross-talk (Cummins, 2001a).

Turning from reproduction, there is expanding recognition of the importance of mitochondrial DNA (mtDNA) deletions and mutations in a range of non-Mendelian-inherited bioenergetic diseases and in normal tissue ageing (Wallace et al., 1995; Ozawa, 1997). To quantify general mitochondrial damage, most publications rely upon the detection of the 4977 bp ‘common deletion’ for convenience, as an unambiguous specific indicator of more general mtDNA damage (Zhang et al., 1996). The deletion arises between tandem 13 bp repeat
sequences through an error in replication. The result is to bring two distant primer target sequences close enough to be amplified by polymerase chain reaction (PCR). This gives a truncated product relative to the intact genome. This is then compared with a control sequence of similar size in a conserved region of the genome, by serial dilution of a DNA sample. Comparison of the dilutions at which the control and deleted products become undetectable is assumed to be an estimate of the relative amounts in the original DNA (Soong and Arnheim, 1996).

There are a number of theoretical and practical problems with this approach, but it persists because of ease of use and the lack of need for specialized equipment. Many groups validate their identification of the deletion using a shift-PCR approach (Zhang et al., 1996) but again the end-point (dilution until product is no longer detectable) means that one is working at the least sensitive point of the PCR reaction. Recently we described a fluorescence-based single-step PCR assay for the equivalent (4834 bp) deletion in rat mtDNA based on competition with a hybrid sequence with binding sites for both. Three-fold dilution series were carried out with the competitor fragment when amplified using deletion primers and the other to measure the amount of control product (total mtDNA) using control primers.

Three-fold dilution series were carried out with the competitor construct and amplified in a PCR reaction using the following 5' fluorescein-labelled primer sets (Gibco BRL, Melbourne, Australia): F'3304–3324F: 5'-AAC ATA CCC ATG GCC AAC CT-3' F'3836–3816R: 5'-GGC AGG AGT AAT CAG AGG TG-3' for the amplification of control products and:

F'8285–8350F: 5'-CTC TAG AGC CCA CTG TAA AG-3'
F'13506–13486R: 5'-GTA GAA ACC TGT GAG GAA AG-3'
for the amplification of deletion products.

Amplification was carried out in 20 µl volumes in 0.2 ml thin-walled tubes (Bresatac, South Australia, Australia), using 20 pmoles of each primer: 300 µmol/l dNTPs; 25 µmol/l MgCl2; 0.7 units Tth+ in 67 mmol/l Tris–HCl; 16.6 mmol/l (NH4)2SO4; 0.45% Triton X-100 and 0.2 mg/ml gelatin (Biotech International, Western Australia, Australia). Cycling was carried out on an Eppendorf Mastercycler. The reaction was started with a denaturation step at 94°C for 5 min, followed by 35 PCR cycles of 94°C for 45 s, 58°C for 45 s and 72°C for 1 min, then a final extension step of 5 min at 72°C.

On completion of amplification, the reaction products were separated on a 2.75% agarose gel in 1× TAE buffer (0.4 M Tris acetate; 0.01 M EDTA, pH 8.3). The products were detected using a Molecular Dynamics Fluorimager SI on a high sensitivity setting at 650 volts and quantified using Imagequant (Molecular Dynamics, Sydney, Australia).

The relative amount of control product compared with deletion (Figure 1) was determined by plotting the log value of the quantity of added competitor DNA (x axis) versus the log value (of the fluorescent signals) of the target divided by the competitor (y axis). Where y = 0.0, the molar ratio of competitor and target are equal and this is extrapolated to the x axis to determine the dilution of competitor at this point. A similar calculation can be performed for both the control and deletion products. The use of the same competitor for both the control and the deletion products means that the amount of deletion present can be expressed as a percentage of the amount of mtDNA present.

We determined that the competitor product amplifies in a linear fashion relative to input DNA and can compete for the respective cloned control and deletion products in a linear fashion. The competitor construct, cloned control and deletion products were quantified using Pico Green (Molecular Dynamics); hence the molar equivalent of the cloned products could be determined. Quantification of the DNA samples allowed the amount of DNA to be added to the reaction to

Materials and methods

Construction of the competitor

The primers listed below were used to amplify the control (predicted size 532 bp) and the deletion (predicted size 244 bp) products from the human mitochondrial genome (Anderson et al., 1981) (excision number: J01415) and to construct the competitor (Figure 1). The control primer set encompasses the NADH subunit 1, and the deletion primer set encompasses a part of the NADH subunit 5 and the tRNAlysine.

The two amplified products were cloned separately into the pCR2.1 vector (Invitrogen, Melbourne, Australia). The heterologous competitor was constructed by shortening the control construct by digesting the plasmid with the enzymes BsmI and HincII, then treating with T4 DNA polymerase I to convert the 5' overhang (generated from BsmI) to a blunt end. The vector was then self-ligated and the resulting control fragment was shown to be 296 bp in length. The deletion fragment was amplified by PCR using the enzyme pfu (Strategene, La Jolla, CA, USA) to give it blunt ends. The control plasmid was then digested with Sall, de-phosphorylated with shrimp alkaline phosphatase and the blunt ended deletion fragment was ligated in.

The whole competitor fragment, including the multiple cloning site of the pCR2.1 vector, was then amplified using standard M13 forward and reverse primers, digested with DelI and SacI, and the ends were treated with T4 DNA polymerase I, to convert the 3' overhangs to blunt ends. Digestion with BamHI and AvaI was then carried out on the fragments and the pCR2.1 vector. The fragments were then ligated into the vector and the final competitor in produces a 453 bp fragment when amplified using the control primers, and a 165 bp fragment with the deletion primers. This gives a product size difference of 79 bp each between the competitor products and the normal product sizes expected when amplifying from human mtDNA. The identities of the final construct and all intermediates were confirmed by sequencing the constructs in both the forward and reverse directions using ABI Prism dRhodamine Terminator and a 310 Genetic Analyser (ABI, Melbourne, Australia).

Serial dilutions method

DNA was extracted from human testicular tissue using standard techniques and mtDNA was estimated using primers and conditions as described previously (Cummins et al., 1998).
Competitive PCR assay for the Δ4977 mt DNA deletion

Figure 1. Diagrammatic representation of the construction of the competitor molecule. The control polymerase chain reaction (PCR) product of 532 bp was cloned into pCR2.1, and an internal fragment was removed by digestion with HincII and BsmI followed by re-ligation. The 244 bp deletion product was cloned into the modified control product by digestion with Stul and ligation. The hybrid molecule was amplified by flanking primers in the vector and an internal fragment removed from the deletion product by digestion with Ddel and Sau96I, followed by re-ligation. The resulting product was cut with BamHI and AvaI and inserted into pCR2.1 to yield competitor-pCR2.1.

be judged and the appropriate dilutions of cloned control and deletion product to be added to the reaction.

To compare the efficiency of the different primer sets, the cloned deletion product was added to a known amount of cloned control product so that it represented 0.1, 1, and 10% of the control product. These mixtures were used to construct a standard calibration curve that relates the output of the deletion curve to the output of the control curve, when the percentage of the deletion is known in the original mixture (Figure 3). Using this relationship, we tested the amount of deletion product in mtDNA samples from human testicular biopsies, where we had previously detected deletions using the serial dilution method (Cummins et al., 1998).

Results

The construction of the competitor (Figure 1) was confirmed by sequencing (data not shown). The final competitor was a 453 bp length fragment in the pCR2.1 vector (Invitrogen), and all primer sites remained intact during the cloning processes. Amplification of the cloned control, deletion and competitor constructs with both sets of primers showed that the predicted size products accumulate in a linear manner, relative to the input sample (Figure 2).

It was evident, however, that the signal intensity from the deleted primer set was higher than that with the normal primer set with the individual cloned products when using equal molar amounts of input DNA. This has been noted previously, and is one of the major limitations of the serial dilution method (Soong and Arnheim, 1996). Therefore, it was necessary to make an adjustment in calculating the amount of deletion in a sample, because when the molar ratios of the products were equal, they did not give the same signal intensity with the different primer sets. The converse was also true. When the signal was equal, the molar ratios of the different products were not equal, as when the same dilution series of competitor

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Figure 2. Determination of the linearity (on a log scale) of competition between (A) control product and (B) deletion product and competitor construct. Known amounts of either cloned normal or cloned deletion product were mixed with diluted competitor molecule. The x axis represents the logarithm value of the concentration of the competitor. In both A and B ‘0’ on the x axis represents an absolute amount of 1.0 pg of competitor DNA. The y axis represents the logarithm of the ratio of fluorescent signal of the added target (either cloned control or deletion product) compared with the competitor. Insert in each case is a picture of the agarose gel from which the data was obtained. In both cases the competition was linear but it was evident that the fluorescent signal differed depending on which primer set was used.

Figure 3. Calibration of the competitive polymerase chain reaction (PCR) assay. Three separate mixtures representing 0.1, 1 and 10% deletion to control product were prepared and assay carried out. The competition observed was plotted for each mixture and a correction factor calculated for each dilution. The mean of this correction factor was then used to standardize the results obtained in the competitive assay, and the results are shown in Table I.

was used for both products. Therefore we tested the competitor to see whether it would compete in a predictable manner with known mixtures of control and deletion products, by adding increasing amounts of competitor to a reaction mix (Figure 2). In both cases, it was evident that the competition was linear. However, it was also evident that, for the same amount of input competitor in either Figure 2A or B (x axis), a substantially different signal ratio was apparent (y axis). Because the signal intensity differed between the primer sets, it was necessary to include a correction factor to calculate the actual amount of deleted product in a mixed population of mtDNAs.

Cloned deletion product was added at 10, 1 and 0.1% of the cloned control product. This 100-fold difference was used to ensure that the assay could be carried out over a wide range of values. The competitive assay was then carried out for each percentage added and the data plotted (Figure 3 and Table I). Knowing the amount of deletion product added, we found that a correction factor of 2.7 was necessary: in other words the molar ratio calculated for the control product must be multiplied by 2.7 to obtain the real ratio between control and deleted.
Table I. Calibration of the quantitative polymerase chain reaction (PCR) assay with known standards

<table>
<thead>
<tr>
<th>Percentage of deleted mtDNA added to mix</th>
<th>Percentage detected by competitive PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.12 ± 0.028</td>
</tr>
<tr>
<td>1.0</td>
<td>1.23 ± 0.24</td>
</tr>
<tr>
<td>10.0</td>
<td>12.25 ± 4.23</td>
</tr>
</tbody>
</table>

To calibrate the assay, known mixes containing the cloned control and deletion products were prepared, so that the deletion represented 0.1, 1 and 10% of the total products. The competitive assay was carried out and a correction value calculated as outlined in the text. The actual amount of deletion calculated in mixes using this single correction value is shown. The variation shown is derived from three separate assays.

Table II. Comparison of the amount of common deletion determined by the serial dilution method and the method described here. Samples including both nuclear and mitochondrial DNA were prepared as previously described for the serial dilution method (Cummins et al., 1998). Figures in parentheses give the exact amounts of mtDNA in terms of the amounts detected relative to the control mtDNA assayed

<table>
<thead>
<tr>
<th>Patient</th>
<th>Serial dilution method</th>
<th>Competitive PCR method</th>
</tr>
</thead>
<tbody>
<tr>
<td>230710</td>
<td>2.43% (0.243/10 ng)</td>
<td>0.00213% (0.008/37.29 pg)</td>
</tr>
<tr>
<td>161117</td>
<td>0.00% (0/100.0 ng)</td>
<td>0.00102% (0.00054/52.78 pg)</td>
</tr>
<tr>
<td>250212</td>
<td>0.24% (0.243/100.0 ng)</td>
<td>0.00224% (0.0083/369.6 pg)</td>
</tr>
<tr>
<td>260614</td>
<td>0.027% (0.037/100.0 ng)</td>
<td>0.00889% (0.0045/51.09 pg)</td>
</tr>
<tr>
<td>071270</td>
<td>0.00% (0/100.0 ng)</td>
<td>0.00010% (0.00007/71.22 pg)</td>
</tr>
<tr>
<td>100819</td>
<td>0.24% (0.243/100 ng)</td>
<td>0.00355% (0.00048/13,515 pg)</td>
</tr>
</tbody>
</table>

The process of age-acquired mtDNA mutations and deletions is still poorly understood. Part of the problem has been that accurate assays, which can measure abnormal to normal ratios in the order of 1 in 10,000, have not been available. In this paper, we describe the construction of a competitive quantitative PCR assay for the Δ4977 mtDNA (Brierley et al., 1998). One group (Moslemi et al., 1996) showed that multiple different deletions of mtDNA may show clonal expansion in individual muscle fibres with increasing age, while other authors (Shoubridge et al., 1990) found focal accumulation of mtDNA mutations may interfere with the normal post-translational expression of wild-type mtDNA. As the average Δ4977 mutation concentration is very low in normal tissues (<1 in 10,000) proper evaluation requires an assay that is both quantitative and accurate at this extreme range.

We found that the serial dilution method seriously overestimated the amount of the common deletion in testicular tissues when compared with our competitive assay. This is probably due to the following reasons. Firstly, the serial dilution approach assumes that an equal input of template yields an equal output of PCR product. This is clearly not the case as originally recognized (Soong and Arnheim, 1996) and as demonstrated with the results reported here and for rat mtDNA (Ahmed et al., 1999). This is because different primer sets give different outputs with the same template. In the data presented here, this was greater than a 10-fold difference with the 0.1% deletion mixture, and with lower proportions of deletions this difference may increase. The difference in product intensity with different primers is most likely due to variations in annealing kinetics, and while primer design may limit this somewhat, it cannot eliminate it altogether. The second reason for the inaccuracies with the serial dilution method is that the end-point is judged on the disappearance of a product, not on an actual value. This will inevitably lead to inaccurate values because the calculation is limited by the sensitivity of detection. In addition, with the serial dilution method, it requires at least three dilution series to estimate the amount of the deletion present. The first of these is to get an estimate of how much total DNA there is present in a particular preparation. The second step is to do a five-fold dilution series and the third to do a two-fold dilution series. Each of these steps is subject to the above limitations and thus errors become multiplied exponentially.

Our competitor method described here overcomes these limitations. Only one dilution series need be carried out and the assay can be standardized with known mixes of DNA. An additional benefit of this is that samples can be compared with each directly, as the same competitor is used and its concentration is known. Therefore, the actual amounts of deleted molecules in a mixture can be calculated. Moreover, the competitor method described here can be standardized and the presence of any impurities or inhibitors in a sample readily identified. While this approach has not completely solved the question of accurate quantification (as discussed above in the need to apply a correction factor), it is far more economical than the ‘gold standard’ of real-time fluorescent quantitative PCR (Orlando et al., 1998; Taylor et al., 2000).

The process of age-acquired mtDNA mutations and deletions is still poorly understood. Part of the problem has been that accurate assays, which can measure abnormal to normal ratios in the order of 1 in 10,000, have not been available. In this paper, we describe the construction of a competitive quantitative PCR assay for the Δ4977 mtDNA...
mutation. With such an assay now available, accurate determination of any increase in this mutation with ageing is now possible. We concentrated here on a series of testicular tissues as we have characterized these samples as accurately as possible using the serial dilution approach. In this study we were unable to dissect out the various components of the testes during DNA extraction to determine if different cell types may be particularly susceptible to the accumulation of deleted mtDNA. If this were possible, it would help us understand whether the amounts of the common deletion found here are relevant to ageing or tissue dysfunction in the testis. Such a study would be particularly relevant given the way in which differential cellular levels of deletions and mutations can lead to widespread tissue dysfunction, as discussed earlier (Shoubridge et al., 1990; Moslemi et al., 1996; Brierley et al., 1998). However, we are confident that the competitor assay will be equally valid for other tissues of interest, as well as for single cells, e.g. oocytes, blastomeres, spermatozoa or polar bodies (Briggs et al., 2000).

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References

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