ORIGINAL ARTICLE

Safarina Malik · Herawati Sudoyo
Patcharin Pramoonjago · Tika Sukarna · Dina Darwis
Sangkot Marzuki

Evidence for the de novo regeneration of the pattern of the length heteroplasmy associated with the T16189C variant in the control (D-loop) region of mitochondrial DNA

Received: October 1, 2001 / Accepted: December 10, 2001

Abstract We have investigated the genetics of the length heteroplasmy associated with the T16189C variant of mitochondrial DNA (mtDNA), and report here definitive evidence that the pattern of the length heteroplasmy is not simply the outcome of drift related to the random segregation of the mtDNA population during cell division, but is actively maintained and regenerated de novo following each cell division. The pattern of the length heteroplasmy was maintained in a fibroblast cell line during an extensive mtDNA depletion experiment by ethidium bromide treatment, and following the subsequent repopulation of the cells with mtDNA. The investigation of the pattern of the length heteroplasmy by single cell pick up shows a similar pattern in sister cells despite the evidence of the randomness of mtDNA segregation, providing definitive evidence for the de novo regeneration of the pattern following cell division. Consistent with this conclusion is the observation that similar patterns of length heteroplasmy are found in tissues of single individuals.

Key words Human mtDNA · D-Loop region · Homopolymeric tract · Length heteroplasmy · De novo regeneration

Introduction

A large body of knowledge has now been accumulated on the role of mitochondrial DNA (mtDNA) mutations as the underlying lesions in a wide range of human neuromuscular and degenerative disorders (see Chinnery et al. 1999; Wallace 1999; Leonard and Schapira 2000 for recent reviews), as well as predisposing factors in polygenic diseases such as diabetes mellitus (Poulton et al. 1998a). The pathogenesis of these disorders is complex and is determined to a

significant extent by the maternal transmission of the mitochondrial genetic factors. The rules of mammalian mitochondrial (cytoplasmic) genetics, however, are still not fully understood. The mammalian mtDNA, encoding 13 polypeptide subunits of the respiratory enzyme complexes, two rRNAs, and 22 tRNAs, exists in cells in multiple copies, ranging, for example, from 800 mtDNA copies/cell in human lung fibroblasts (Robin and Wong 1988) to 6000 mtDNA copies/cell in skin fibroblasts (Shmookler Reis and Goldstein 1983). These mtDNA molecules segregate randomly during cell division (Lertrit et al. 1992; Marzuki et al. 1997; Meirelles and Smith 1998), but several recent observations strongly suggest that the outcome of this segregation process is probably modulated by other factors. Thus, for example, despite the high rate of mutation in the mtDNA (5–10 times higher than that of the nuclear DNA; Brown et al. 1979), the expected heteroplasmic state of the mtDNA, i.e., the existence of more than one population of mtDNA within a cell, is rarely found.

Complete switching of the mtDNA genotype has been demonstrated to occur within one or two generations in Holstein cows that are heteroplasmic for a neutral mtDNA genotype; this observation led to a proposal of the existence of a genetic bottleneck for the mtDNA in the female germ line or in the early embryo (Hauswirth and Laipis 1982). The effective number of the mtDNA segregating unit in mice during early oogenesis is approximately 200 (Jenuth et al. 1996), while for humans it ranges from one to 135 (Marchington et al. 1997; Poulton et al. 1998b). Perhaps, superimposing on the germ-line bottleneck is the possibility that not all copies of mtDNA in a cell participate equally in the mtDNA replication (Tourte et al. 1984; D'Herde et al. 1995) or segregation (Meirelles and Smith 1998) processes. Further, certain mtDNA mutations might confer selective advantage/disadvantage on the cells, and this factor could contribute significantly to the outcome of mtDNA segregation. In pedigrees with Leber's hereditary optic neuropathy (LHON), segregation favoring the mutant (G11778A) mtDNA type is relatively rapid (Savontaus 1995), while in mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) and myoclonic epilepsy with

S. Malik · H. Sudoyo · P. Pramoonjago · T. Sukarna · D. Darwis · S. Marzuki (✉)
Eijkman Institute for Molecular Biology, Jl. Diponegoro 69, Jakarta 10430, Indonesia
Tel. +62-21-3148694; Fax +62-21-3147982
e-mail: smarzuki@eijkman.go.id

ragged-red fibers (MERRF), the heteroplasmic state is maintained for several generations (Chinnery et al. 1998).

Several homopolymeric tracts in the mtDNA exhibit length polymorphisms, i.e., the presence in cells of multiple mtDNA species with various lengths of the homonucleotide run. The genetic characteristics of these length polymorphisms have been the subject of investigations to gain insights into the factors that influence the transmission of the mtDNA, in particular in relation to the germ-line bottleneck (Hauswirth et al. 1984; Marchington et al. 1997). One of the homopolymeric tracts, which is associated with a mtDNA T16189C variant located within and interrupting a homopolymeric tract of cytosines between nt16184 and nt16193 (Bendall and Sykes 1995; Marchington et al. 1996), shows a prominent deviation from the common rules that govern mtDNA transmission, and is thus of great interest. This variant has been suggested to be associated with several common human diseases such as diabetes mellitus (Poulton et al. 1998a), low birth weight (Casteels et al. 1999), and dilated cardiomyopathy (Khogali et al. 2001), and thus is also of medical significance.

The T16189C mutation results in an unstable homopolymeric tract of ten cytosines, and length variation of the poly [C] is observed in individuals in a heteroplasmic manner (i.e., length variants of between 8 and 14 cytosines are found in one individual) (Bendall and Sykes 1995). The relative proportion of the various lengths of poly [C] (pattern of length heteroplasmy) associated with the T16189C variant appears to be maintained during cell division (Bendall and Sykes 1995; Marchington et al. 1996). Thus, while the pattern of the length heteroplasmy differs in different individuals, this pattern appears to be similar in different tissues from an individual (Marchington et al. 1996), suggesting that the segregation of the length variants is not random. Further, maternally related members of a family appear to maintain the same pattern of the length heteroplasmy. There is a compelling need to confirm that the pattern of the length heteroplasmy associated with the T16189C variant is indeed actively maintained during cell division, as it would imply that there must be factors that regulate the generation of this heteroplasmic pattern. We have thus investigated the genetics of the length heteroplasmy associated with the T16189C variant, and report here definitive evidence for the de novo regeneration of the pattern of the length heteroplasmy following cell division.

Materials and methods

Fibroblast cell lines, blood, and tissues

Two fibroblast cell lines were employed, one (EISbIII-31-95) derived from a member of a family with Leber's hereditary optic neuropathy (LHON) reported previously (EISb-95; Sudoyo et al. 1998), and one (EIN-3-95) derived from a normal individual (Malik et al. 2000). For the generation of EISbIII-31-95, a skin biopsy was taken with informed consent as part of the diagnostic work up of the

patient. Procedures for the biopsy and the generation of fibroblast cell lines are as previously reported (Malik et al. 2000). Blood samples from normal individuals were taken with informed consent.

Ten postmortem tissue samples (thyroid gland, liver, diaphragm, psoas muscle, kidney, adrenal gland, heart, cerebral cortex, basal ganglia, and white matter) were obtained at the Cipto Mangunkusumo Hospital morgue, Jakarta, Indonesia, from each of five normal individuals who died from nonpathological causes. Autopsies were carried out according to standard technical, legal, and ethical procedures of the Cipto Mangunkusumo Hospital. Tissue samples were stored individually in small containers at -80°C until used.

DNA extraction and detection of the mtDNA T16189C variant

Total DNA from blood and fibroblast cells was extracted as described elsewhere (Lertrit et al. 1992). DNA from tissues was extracted by an organic extraction method (Sambrook et al. 1989) with modifications. A small piece of tissue was homogenized in a tight-fit glass-Teflon homogenizer, in the presence of a cell lysis buffer containing 10mM Tris-HCl (pH 8.0), 25mM ethylenediamine tetraacetate (EDTA), and 0.5% sodium dodecyl sulphate (SDS). To the lysate, 50 $\mu\text{g}/\text{ml}$ RNase T (Qiagen, Hilden, Germany) was added, followed by incubation at 37°C for 30min. Protein was then precipitated with ammonium acetate and chloroform (1:1). Following ethanol precipitation, DNA was resuspended in a 10mM Tris-HCl buffer containing 1mM EDTA (pH 8.0).

A 637-bp fragment of the mtDNA between nt15904 and nt16540 (Cambridge reference sequence; Anderson et al. 1981) was amplified by polymerase chain reaction (PCR) by using the primer pair L15904 (5'-CTAATACACCAGTCTTGTAACCGGAG-3') and H16540 (5'-GTGGGCTATTAGGCTTTATGACCCTG-3') in a programmable thermal cycler (Perkin Elmer 9700 GeneAmp System; ABI-PE, Norwalk, CT, USA), essentially as described by Saiki et al. (1985). The PCR was carried out for 35 cycles of denaturation at 95°C for 20s (first cycle at 95°C for 5min), annealing at 62°C for 20s, and elongation at 72°C for 1min. The amplification product was then digested with *MnII* restriction endonuclease (New England Biolabs, Beverly, MA, USA), and the fragments were separated by electrophoresis on a 2% agarose gel. The T to C base substitution at nt 16189 was detected as the loss of an *MnII* restriction site (Bendall et al. 1996; Marchington et al. 1996).

Quantitation of the poly [C] length variant

A procedure was developed to quantitate the poly [C] length variants associated with the T16189C polymorphism. A 172-bp fragment spanning nt16142 to nt16313 of the mtDNA was amplified by PCR by using the primer pair L16142 (5'-CTTGACCACCTGTAGTACAT-3') and H16313 (5'-GTACTATGTACTGTAAAGGG-3'). To

avoid artifactual length variations due to 1-bp T-overhangs introduced by the *Taq* polymerase at the 3' end that would otherwise complicate the analysis of the length of the poly [C], a restriction endonuclease (*RsaI*, Promega, Madison, WI, USA) was employed to trim both ends of the PCR products (Marchington et al. 1996) resulting in blunt-end fragments of 50–56 bp, depending on the length of the poly [C]. The relative proportions of the various length variants were quantitated using a Bio-Rad GS-700 imaging densitometer, after the separation of the trimmed PCR products in a 20% polyacrylamide gel (run at 2300 V for 4 h) and visualization of the DNA fragments by either silver staining or autoradiography of (³³P)-radiolabeled products (details and validation are described in Results).

Reduction and quantitation of mtDNA copy number

Fibroblast cells (EIN-3-95) were cultured in the presence of 50 ng/ml ethidium bromide essentially as described previously (King and Attardi 1989; Vaillant and Nagley 1995). The reduction of the mtDNA copy number was assessed during this process by employing a quantitative PCR method, which was carried out with internal standards for both mtDNA and nuclear DNA. The internal standard for the mtDNA was a 602-bp mtDNA fragment spanning nt2055–nt2656, which had been inserted into the pGEMT-easy vector (Promega). This fragment carried a PCR-introduced *BglI* restriction site, which was not present in the normal mtDNA, and thus allowed the differentiation of the PCR product of the internal standard from that of the mtDNA being examined. The internal standard for the nuclear DNA was a pGEMT-easy vector with a 123-bp PCR-amplified insert of the $\alpha 1$ -globin gene, which had lost the *HinfI* restriction site at codon 116. This mutation is rare and found only in individuals with Hemoglobin O Indonesia (HbO_{Indo}), which is restricted to the populations of South Sulawesi (Daud et al. 2001), and thus should not be present in the samples examined in the current study. Fixed amounts (2 pg) of the internal standards for mtDNA and nuclear DNA were coamplified with the DNA samples in serially diluted aliquots of the fibroblast lysates (Zhang et al. 1996; Filser et al. 1997).

For the quantitation of the mtDNA, a 289-bp fragment was amplified in a Perkin Elmer 9700 thermal cycler by employing the primer pair L2055 (5'-TGCCACAGAAC CCTCTAAATCCC-3') and H2343 (5'-CAGGCTTATGC GGAGGAGAATGTT-3'). The amplification was carried out for 28 cycles of denaturation for 20 s at 95°C (first cycle 5 min at 95°C), annealing for 20 s at 62°C, elongation for 1 min at 72°C (last cycle 5 min at 72°C), followed by 5 min at 95°C and 5 min at 25°C for final denaturation and slow annealing. The quantitation of the nuclear DNA was performed by using the primer pair HbO and Alf1 (Daud et al. 2001) resulting in a 123-bp fragment. The PCR amplification was carried out for 34 cycles of denaturation at 95°C for 30 s (the first cycle 95°C for 5 min), annealing and elongation at 70°C for 30 s (first cycle 70°C for 1 min; last cycle 70°C for 5 min), followed by final denaturation at

95°C for 5 min, and slow annealing at 25°C for 5 min. The PCR products were then digested with either *BglI* restriction endonuclease (Gibco/BRL, Rockville, MD, USA) for mtDNA quantitation, or *HinfI* restriction endonuclease (New England Biolabs) for nuclear DNA quantitation. The digested products were separated by electrophoresis on a 2% agarose gel, and the intensity of the ethidium bromide-stained fragments were quantitated by densitometric analysis as described above. The copy number of the mtDNA was calculated by comparing the intensity of the digested PCR products of the internal standards with the undigested PCR products of the samples being examined for mtDNA quantitation, and vice versa for the nuclear DNA quantitation. The copy number quantitation was carried out in triplicate at the exponential stage of the amplification. Corrections were made for the length-dependent variation of the ethidium bromide staining intensity, and for heteroduplex formation.

Single cell PCR

Fibroblast cells (EISbIII-31-95 carrying the heteroplasmic T14484C LHON mutation and the 16189C variant, and EIN-3-95 carrying only the 16189C variant) were grown in very low cell densities of approximately ten cells per 35-mm tissue culture dish (Nunc, Naperville, IL, USA). After the first cell division (48–72 h), the attached cells were washed twice with phosphate-buffered saline (PBS; Sambrook et al. 1989) containing a low concentration of 0.0125% (w/v) trypsin. Single fibroblast cells were picked up under an inverted microscope (Nikon, Tokyo, Japan) by suction by using borosilicate microcapillary tubes with an approximately 3- μ m-diameter opening, connected to a micromanipulator (Narishige, Tokyo, Japan) (Bidooki et al. 1997). Each isolated cell was delivered directly into a 0.2-ml microfuge tube containing 5 μ l sterile double-distilled water, lysed at 95°C for 10 min, and subjected to nested PCR amplification.

The first PCR amplified a 637-bp (with primer pair L15904-H16540) and an 897-bp (with primer pair L13977, 5'-CCTGCCCCCTACTCCTCCTAGACCT-3' and H14873, 5'-GGATCAGGCAGGCGCCAAGGAGTG-3') fragment of the mtDNA. The second PCR to detect the 16189C variant, amplified a 172-bp fragment between nt16142 and nt16313, as described above. The T14484C mutation was detected and quantitated essentially as previously described (Sudoyo et al. 1998).

Results

Features of the mtDNA length heteroplasmy

We first need to confirm the main features of the mtDNA length heteroplasmy associated with the T16189C polymorphism. This polymorphic site is located within and interrupts a homopolymeric tract of cytosines between nt16184

and nt16193, and the 16189C variant is associated with the heteroplasmic presence of mtDNA species carrying different lengths of poly [C]. For the quantitation of the length heteroplasmy, we have developed a procedure based on the T-PCR (trimmed-PCR) principle described by Marchington et al. (1996). Electrophoresis of the trimmed-PCR products in a high concentration polyacrylamide gel separates the length variants associated with the H- and L-strands of the mtDNA fragments, allowing duplicate quantitative measurements from a single run (Fig. 1a). The variants associated with the expected 52-bp PCR-amplified fragment could be clearly detected, which in the case illustrated ranged from 51–54 bp (poly [C] length 11–14; Fig. 1a). To ensure that the quantitative method developed is reliable, a 410-bp PCR product spanning nt15904 to nt16313 containing the poly [C] tract was cloned into the M13 vector (Perkin Elmer, Branchburg, NJ, USA). The relative proportions of the mtDNA species carrying different lengths of poly [C] was determined by the sequencing of 75 clones. The pattern of the length heteroplasmy obtained by our quantitative procedure was essentially similar to this gold standard (Fig. 1b), thus validating the T-PCR method. Further, PCR amplification of the cloned mtDNA fragments showed that each clone gave only a single poly [C] length, confirming that the length heteroplasmy observed in tissues was not an artifact generated by the PCR process.

We confirmed that the relative proportions of the various lengths of poly [C] vary between individuals, as illustrated for four individuals in Fig. 2; one with the ¹⁶¹⁸⁰AAAACCCCCCCCC¹⁶¹⁹³ variant, one with the ¹⁶¹⁸⁰AAACCCCCCCCC¹⁶¹⁹³ variant with an additional T16183C substitution, and two individuals with the ¹⁶¹⁸⁰AACCCCCCCCC¹⁶¹⁹³ variant with a further T16182C substitution.

The pattern of the length heteroplasmy is actively maintained

To obtain initial experimental evidence that the length heteroplasmy observed is not simply the outcome of drift associated with the random segregation of the mtDNA population during cell division but is actively maintained, an experiment was designed to follow the reestablishment of the pattern during the repopulation of depleted cells with mtDNA. The mtDNA copy number in a normal fibroblast cell line (EIN-3-95) was extensively reduced to approximately 85 copies/cell, about one-fortieth of the original copy number of approximately 3300 copies/cell, by culturing in the presence of 50 ng/ml ethidium bromide for approximately 15 cell divisions. The cells were then released from ethidium bromide exposure to allow the repopulation of the cells with mtDNA. The pattern of the length heteroplasmy remains similar during the reduction of the mtDNA copy number (i.e., 6, 10, and 15 cell divisions in the presence of ethidium bromide; Fig. 3a–c). When the pattern was examined after the cell repopulation with mtDNA (i.e., approx. 15 further cell divisions following the release from the ethidium bromide treatment), the pattern was found to

Fig. 1. The relative proportion of the various lengths of poly [C] associated with the mtDNA 16189C variant. The pattern of the *poly [C]* length heteroplasmy was determined by polymerase chain reaction (PCR) amplification of a 172-bp fragment spanning nt16142 to nt16313 of the mtDNA, and digestion by the *RsaI* restriction endonuclease to trim the 5' and 3' ends of the products (**a top**). The trimmed-PCR (T-PCR) products were separated by electrophoresis in a 20% polyacrylamide gel, and the length variants (51–54 bp) associated with the H- and L-strands were visualized by silver staining (**a middle**). The relative proportions of the various variants were quantitated using a Bio-Rad GS-700 imaging densitometer with Molecular Analyst software version 2.1 (**a bottom**). The validity of this quantitative method was confirmed as shown in **b**. A 410-bp PCR-amplified fragment spanning nt15904 to nt16313 containing the poly [C] tract was cloned into the M13 vector. Shown is the pattern of the length heteroplasmy as determined by the direct sequencing of the cloned mtDNA fragments (75 clones; *black*), and those obtained by our T-PCR method after visualization of the trimmed-PCR products by either silver staining (*white*) or autoradiography of (³²P)-labeled products (*gray*). Results shown for T-PCR are the average (±SD) of at least three independent duplicate measurements. Please note that the patterns of the length heteroplasmy in **a** and **b** are from two different individuals and thus exhibit different patterns

Fig. 2. The pattern of the length heteroplasmy in four individuals. The proportions of the various length variants (i.e., patterns of the length heteroplasmy) associated with the 16189C variant were determined in four individuals as in Fig. 1. Shown are the nucleotide sequences around the T16189C site revealed by sequencing and the associated pattern of the length heteroplasmy. One individual had the ¹⁶¹⁸⁰AAAACCCCCCCCC¹⁶¹⁹³ variant, one had the ¹⁶¹⁸⁰AAACCCCCCCCC¹⁶¹⁹³ variant, with the T16183C substitution, and two had the ¹⁶¹⁸⁰AACCCCCCCCC¹⁶¹⁹³ variant with a further T16182C substitution

be similar also to that of the untreated cells (Fig. 3d), indicating de novo regeneration of the pattern of the length heteroplasmy.

The pattern of the length heteroplasmy is regenerated de novo following cell division

Definitive evidence for the de novo regeneration of the pattern of the length heteroplasmy was obtained by directly investigating the pattern in sister cells following cell division of single cells. The initial study on fibroblast cell line EIN-3-95 showed that in all four pairs of single cells, the pattern of the length heteroplasmy was similar in sister cells (Fig. 4).

MtDNA is randomly segregated during cell division, so to confirm the randomness of the segregation, we employed another cell line, which carried a heteroplasmic T14484C mtDNA mutation. Fig. 5 shows the results of two such experiments. In the first, the two sister cells had almost identical ratios (11:89 and 13:87) of the 14484T to 14484C mtDNA species. The pattern of the length heteroplasmy in the two sister cells was also essentially the same. In the second pair of cells, random segregation of the mtDNA was observed prominently (Fig. 5, pair 2). Thus, while in one cell the 14484T to 14484C ratio was 46:54, the ratio in the other

Table 1. Pattern of poly [C] length heteroplasmy in sister cells

	Mean length of poly [C]	14484T/14484C (%)
I-1	11.7 ± 0.96	11/89
I-2	11.9 ± 1.09	13/87
II-1	11.7 ± 0.92	46/54
II-2	11.6 ± 1.01	100/0
III-1	11.8 ± 1.20	98/2
III-2	11.4 ± 0.63	16/84
IV-1	11.4 ± 1.14	93/7
IV-2	11.3 ± 1.07	18/82
V-1	11.2 ± 1.20	28/72
V-2	11.6 ± 0.93	21/79
VI-1	11.3 ± 1.16	11/89
VI-2	11.4 ± 0.96	19/81
VII-1	11.4 ± 0.64	21/79
VII-2	11.4 ± 0.72	13/87
VIII-1	10.9 ± 0.83	10/90
VIII-2	11.5 ± 1.03	16/84
IX-1	11.3 ± 0.96	15/85
IX-2	10.8 ± 1.03	0/100
X-1	11.2 ± 0.80	8/92
X-2	11.1 ± 1.00	6/94
XI-1	11.3 ± 1.37	85/15
XI-2	11.1 ± 0.89	5/95
XII-1	11.2 ± 0.75	32/68
XII-2	11.3 ± 1.02	13/87

Results are expressed as the mean length of poly [C] ± SD of at least three independent measurements

Fig. 3. The patterns of the length heteroplasmy in a fibroblast cell line during mtDNA copy number reduction and following repopulation. The mtDNA copy number in a normal fibroblast cell line (EIN-3-95) was extensively reduced by culturing the cells in the presence of 50 ng/ml ethidium bromide (EtBr) for approximately 15 cell divisions. The mtDNA copy number was assessed by quantitative PCR essentially as described in Material and methods, and was approximately 3300 copies/cell in the untreated cells and 85 copies/cell after 15 cell divisions in ethidium bromide. The cells were then released from the ethidium bromide exposure to allow the repopulation of the cells with

mtDNA. The pattern of the length heteroplasmy was determined before (a) and during the reduction of the mtDNA copy number [at 6 (not shown, but the pattern was similar to that in a), 10 (b), and 15 (c) cell divisions in the presence of ethidium bromide]. Shown in d is the pattern of the length heteroplasmy determined after the ethidium bromide removal from the culture to allow the cell repopulation with mtDNA (i.e., approx. 15 further cell divisions). The relative proportion of the various length variants was determined as in Fig. 1. Results are the mean $\pm$ SD of three independent duplicate experiments

cell was 100:0. Despite this significant drift, the pattern of the length heteroplasmy was still essentially the same in the two sister cells. The results obtained from 12 pairs of single cells are summarized in Table 1. In all cases, the mean length of the poly [C] was essentially the same, with an average value of 11.4 ± 0.28 , despite the fact that the ratio of the 14484T- to the 14484C-carrying mtDNA species varied widely from 100:0 to 0:100. This result provides definitive evidence in support of the suggestion that the pattern of the length heteroplasmy is regenerated de novo.

The pattern of the length heteroplasmy is similar in tissues of an individual

Finally, we have argued that if the pattern of the length heteroplasmy is indeed regenerated de novo, independent

of the random segregation of mtDNA, the pattern of the length heteroplasmy would then be the same in all tissues of an individual. A limited set of results is indeed consistent with the above argument (Marchington et al. 1996, 1997). To obtain stronger evidence in support of this suggestion, we carried out an extensive examination involving ten different postmortem tissue samples (thyroid gland, liver, diaphragm, psoas muscle, kidney, adrenal gland, heart, cerebral cortex, basal ganglia, and white matter) for each of five individuals. The pattern of the length heteroplasmy varied among these five individuals, but similar, although not completely identical, patterns were observed in all tissues of each individual (illustrated in Fig. 6).

Fig. 4. The patterns of the length heteroplasmy in sister cells of fibroblasts EIN-3-95. Fibroblast cells (EIN-3-95) carrying the 16189C variant were grown in very low cell densities of approximately ten cells per 35-mm-tissue culture dish. After the first cell division (48–72 h), single fibroblast cells grown next to each other were picked up. Each isolated cell was subjected to nested PCR amplification and restriction enzyme digestion as in Fig. 1 to determine the pattern of the length heteroplasmy. Shown are the results for four pairs of cells, which demonstrate a similar pattern of length heteroplasmy

Fig. 5. The patterns of the length heteroplasmy in sister cells of fibroblasts EISbIII-31-95. A cell line carrying the T16189C variant and a heteroplasmic T14484C mtDNA mutation as a marker for mtDNA random segregation was used. Fibroblast cells were grown and picked up singly, and the pattern of the length heteroplasmy was determined as in Fig. 3. Shown are two of twelve pairs examined

Discussion

We presented here evidence to answer the question on whether the pattern of the length heteroplasmy associated with the T16189C variant is simply the outcome of drift associated with the random segregation of the mtDNA

population during cell division, or whether this pattern is actively maintained and regenerated de novo following each cell division. Three independent experimental approaches were employed. The first approach was designed to mimic the bottleneck proposed to exist in mtDNA segregation during early oogenesis. The mtDNA copy number was thus reduced significantly from around 3300 to 85

Fig. 6. The patterns of the length heteroplasmy in various tissues. Ten different postmortem tissue samples (thyroid gland, liver, diaphragm, psoas muscle, kidney, adrenal gland, heart, cerebral cortex, basal ganglia, and white matter) were obtained from each of five individuals at

autopsy. The relative proportion of the various length variants associated with the 16189C variant was determined as in Fig. 1. A representative set of data from one individual is shown. In all five cases, a similar pattern of length heteroplasmy was observed in all tissues

copies/cell by exposure to ethidium bromide (King and Attardi 1989; Vaillant and Nagley 1995); the fibroblast cells were then allowed to divide for 15 generations starting from this low mtDNA copy number following the release from the ethidium bromide treatment. The results of this study demonstrated the maintenance of the pattern of the length heteroplasmy during the reduction of the mtDNA copy number and the subsequent cell repopulation with mtDNA, suggesting the *de novo* regeneration of the pattern. The investigation of the pattern of the length heteroplasmy in sister cells following cell division in the second approach provided definitive evidence for the *de novo* regeneration of the pattern. In this study, we were able to demonstrate directly that the pattern of the length heteroplasmy was maintained despite the evidence for a significant randomness of mtDNA segregation. Further, we confirmed that the pattern of the length heteroplasmy was indeed regenerated *de novo*, independent of the random segregation, as similar patterns were observed in as many as ten tissues from each of five individuals. To ensure that the pattern of the length heteroplasmy observed was not an artifact caused by polymerase slippage during PCR amplification, we cloned fragments containing the poly [C] tract associated with the T16189C variant and demonstrated that only a single length variant was apparent in each of the 75 clones examined.

Our finding that the pattern of the length heteroplasmy is regenerated *de novo* has an important implication in that there must be factors that regulate the regeneration of this pattern. Earlier studies have indicated that the pattern of the length heteroplasmy associated with the 16189C variant is maintained in the maternal line (Marchington et al. 1996; Bendall et al. 1996). Because this implies that the pattern of the length heteroplasmy cosegregates with the mtDNA,

which is maternally inherited, the pattern is most likely determined by factors within the mtDNA. We examined the pattern of the length heteroplasmy in three independent families and confirmed that while the pattern was different in the three families, maternally related members of each family showed similar patterns of the length heteroplasmy (data not shown).

What are the factors responsible for the maintenance of the pattern of the length heteroplasmy? Sequence polymorphisms in the mtDNA appear to be involved either in the phenotypic expression of the pathological mtDNA mutations, or in their actual generation, as indicated by the clustering of the LHON G11778A mutation in certain lineages of the mtDNA (haplotype groups; haplogroups) (Brown et al. 1997; Lamminen et al. 1997; Torroni et al. 1997; Sudoyo H., Suryadi H., Lertrit P., Pramoonjago P., Lyrwati D., Marzuki S., unpublished). Such polymorphisms could also be the responsible mtDNA factors that maintain the pattern of the length heteroplasmy.

We suspect, however, that the generation of the length heteroplasmy associated with homopolymeric tracts in the mtDNA is probably also under the influence of nuclear factors in addition to the mitochondrial factors. The mtDNA replication machinery appears to be able to replicate homopolymeric tracts faithfully up to the length of six cytosines, as observed in the homopolymeric tract associated with the T16189C/C16187T variants, which did not show any length variation (Bendall and Sykes 1995; Howell and Smejkal 2000). At a poly [C] length of seven or eight (Howell and Smejkal 2000), however, the length heteroplasmy could be observed, but only in certain individuals, indicating the existence of population variants in the fidelity of the nuclear coded mtDNA replication

machinery. The study of the interplay between the nuclear and mitochondrial factors in the generation of the length heteroplasmy would lead to a better understanding of the factors that influence the development of mtDNA mutations.

Acknowledgments This work was supported by grants from PT Krakatau Steel and PT Inti through the Agency for Strategic Industries (Indonesia) and by a generous development fund from the National Development Planning Agency (BAPPENAS) of the Republic of Indonesia. Part of the work is included in S. Malik's thesis, submitted to Monash University, Melbourne, Australia, for a Ph.D. degree. She thanks Dr. T.W. Spithill for his support as her academic supervisor.

References

- Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG (1981) Sequence and organization of the human mitochondrial genome. *Nature* 290:457–465
- Bendall KE, Sykes BC (1995) Length heteroplasmy in the first hypervariable segment of the human mtDNA control region. *Am J Hum Genet* 57:248–256
- Bendall KE, Macauley VA, Baker JR, Sykes BC (1996) Heteroplasmic point mutations in the human mtDNA control region. *Am J Hum Genet* 59:1276–1287
- Bidooki SK, Johnson MA, Chrzanowska-Lightowlers Z, Bindoff LA, Lightowlers RN (1997) Intracellular mitochondrial triplasm in a patient with two heteroplasmic base changes. *Am J Hum Genet* 60:1430–1438
- Brown WM, George M Jr, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. *Proc Natl Acad Sci U S A* 76:1967–1979
- Brown MD, Sun F, Wallace DC (1997) Clustering of Caucasian Leber hereditary optic neuropathy patients containing the 11778 or 14484 mutations on an mtDNA lineage. *Am J Hum Genet* 60:381–387
- Casteels K, Ong K, Phillips D, Bendall H, Pembrey M (1999) Mitochondrial 16189 variant, thinness at birth, and type-2 diabetes. ALSPAC study team. *Avon Longitudinal Study of Pregnancy and Childhood. Lancet* 353:1499–1500
- Chinnery PF, Howell N, Lightowlers RN, Turnbull DM (1998) MELAS and MERRF. The relationship between maternal mutation load and the frequency of clinically affected offspring. *Brain* 121:1889–1894
- Chinnery PF, Howell N, Andrews RM, Turnbull DM (1999) Clinical mitochondrial genetics. *J Med Genet* 36:425–436
- Daud D, Harahap A, Setianingsih I, Nainggolan I, Trangana S, Pakasi R, Marzuki S (2001) Hemoglobin O mutation in Indonesia: distribution and phenotypic expression. *J Med Genet* 46:499–505
- D'Herde K, Callebaut M, Roels F, De Prest B, van Nassauw L (1995) Homology between mitochondriogenesis in the avian and amphibian oocyte. *Reprod Nutr Dev* 35:305–311
- Filser N, Margue C, Richter C (1997) Quantification of wild-type mitochondrial DNA and its 4.8kb deletion in rat organs. *Biochem Biophys Res Commun* 223:102–107
- Hauswirth WW, Laipis PJ (1982) Mitochondrial DNA polymorphism in a maternal lineage of Holstein cow. *Proc Natl Acad Sci U S A* 72:4686–4690
- Hauswirth WW, Van de Walle MJ, Laipis PJ, Olivo PD (1984) Heterogenous mitochondrial DNA D-loop sequence in bovine tissue. *Cell* 37:1001–1007
- Howell N, Smejkal CB (2000) Persistent heteroplasmy of a mutation in the human mtDNA control region: hypermutation as an apparent consequence of simple-repeat expansion/contraction. *Am J Hum Genet* 66:1589–1598
- Jenuth JP, Peterson AC, Fu K, Shoubridge EA (1996) Random genetic drift on the female germline explains the rapid segregation of mammalian mitochondrial DNA. *Nat Genet* 14:146–151
- Khogali SS, Mayosi BM, Beattie JM, McKenna WJ, Watkins H, Poulton J (2001) A common mitochondrial DNA variant associated with susceptibility to dilated cardiomyopathy in two different populations. *Lancet* 357:1265–1267
- King MP, Attardi G (1989) Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science* 246:500–503
- Lamminen T, Huoponen K, Sistonen P, Juvonen V, Lahermo P, Aula P, Nikoskelainen E, Savontaus ML (1997) MtDNA haplotype analysis in Finnish families with Leber hereditary optic neuroretinopathy. *Eur J Hum Genet* 5:271–279
- Leonard JV, Schapira AHV (2000) Mitochondrial respiratory chain disorder I: mitochondrial DNA defects. *Lancet* 355:299–304
- Lertrit P, Noer AS, Byrne E, Marzuki S (1992) Tissue segregation of a heteroplasmic mtDNA mutation in MERRF (myoclonic epilepsy with ragged red fibers) encephalomyopathy. *Hum Genet* 90:251–254
- Malik S, Sudoyo H, Marzuki S (2000) Microphotometric analysis of NADH-tetrazolium reductase deficiency in fibroblasts of patients with Leber's hereditary optic neuropathy. *J Inher Metab Dis* 23:730–744
- Marchington DR, Hartshorne GM, Barlow D, Poulton J (1996) Do sequence variants in the major non-coding region of the mitochondrial genome influence mitochondrial mutations associated with disease? *Hum Mol Genet* 5:473–479
- Marchington DR, Hartshorne GM, Barlow D, Poulton J (1997) Homopolymeric tract heteroplasmy in mtDNA from tissues and single oocytes: support for a genetic bottleneck. *Am J Hum Genet* 60:408–416
- Marzuki S, Berkovic SF, Noer AS, Kapsa RM, Kalnins RM, Byrne E, Sasmono T, Sudoyo H (1997) Developmental genetics of deleted mtDNA in mitochondrial oculomyopathy. *J Neurol Sci* 145:155–162
- Mereilles FV, Smith LC (1998) Mitochondrial genotype segregation during preimplantation in mouse heteroplasmic embryos. *Genetics* 148:877–884
- Poulton J, Brown MS, Cooper A, Marchington DR, Phillips DI (1998a) A common mitochondrial DNA variant is associated with insulin resistance in adult life. *Diabetologia* 41:51–58
- Poulton J, Macaulay V, Marchington DR (1998b) Mitochondrial genetics '98: is the bottleneck cracked? *Am J Hum Genet* 62:752–757
- Robin ED, Wong R (1988) Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. *J Cell Phys* 136:507–513
- Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1985) Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350–1354
- Sambrook J, Fritsch EF, Manniatis T (1989) *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor, New York
- Savontaus ML (1995) MtDNA mutations in Leber's hereditary optic neuropathy. *Biochim Biophys Acta* 1271:261–263
- Shmookler Reis RJ and Goldstein S (1983) Mitochondrial DNA in mortal and immortal human cells. *J Biol Chem* 258:9078–9084
- Sudoyo H, Sitepu M, Malik S, Poesponegoro HD, Marzuki S (1998) Leber's hereditary optic neuropathy in Indonesia: two families with the mtDNA 11778G > A and 14484T > C mutations. *Hum Mutat Suppl* 1:S271–S274
- Torroni A, Petrozzi M, D'Urbano L, Sallito D, Zeviani M, Carrara F, Carducci C, Leuzzi V, Carelli V, Barboni P, De Negri A, Scozzari R (1997) Haplotype and phylogenetic analyses suggest that one European-specific mtDNA background plays a role in the expression of Leber hereditary optic neuropathy by increasing the penetrance of the primary mutations 11778 and 14484. *Am J Hum Genet* 60:1107–1121
- Tourte M, Mignotte F, Mounolou JC (1984) Heterogeneous distribution and replication activity of mitochondria in *Xenopus laevis* oocytes. *Eur J Cell Biol* 34:171–178
- Vaillant F, Nagley P (1995) Human cell mutants with very low mitochondrial DNA copy number (rho d). *Hum Mol Genet* 4:903–914
- Wallace DC (1999) Mitochondrial diseases in man and mouse. *Science* 283:1482–1488
- Zhang C, Peters LE, Linnane AW, Nagley P (1996) Comparison of different quantitative PCR procedures in the analysis of the 4977-bp deletion in human mitochondrial DNA. *Biochem Biophys Res Commun* 223:450–455