Vol. 164, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1989 Pages 156-163

DIRECT SEQUENCING OF DELETED MITOCHONDRIAL DNA IN MYOPATHIC PATIENTS

Masashi Tanaka, Wataru Sato, Kinji Ohno, Tomoko Yamamoto, and Takayuki Ozawa

Department of Biomedical Chemistry, Faculty of Medicine,
University of Nagoya, Nagoya 466, Japan

Received August 16, 1989

Summary: **To investigate the mechanism of mitochondrial DNA deletion in human diseases, we amplified the deleted mitochondrial DNA of five patients with mitochondrial myopathy by using the polymerase chain reaction, and directly sequenced the crossover regions of the deleted mitochondrial DNA without cloning. In Patient 1, a 7-bp directly repeated sequence of 5'-ATCCCCA-3' was found at the boundaries of deleted segment spanning 7,039 bp between the ATPase 6 and the cytochrome *b* genes. In Patients 2, 3, and 4, a 13-bp sequence of 5'-ACCTCCCTCACCA-3' was found in the boundaries of deleted segment spanning 4,977 bp between the ATPase 8 and the ND5 genes. In Patient 5, a 3-bp sequence of 5'-CCT-3' was found in the boundaries of deleted segment spanning 3,717 bp between the ATPase 6 and the ND5 genes. Similar directly repeated sequences may contribute to mitochondrial DNA deletions in human degenerative diseases.**

Human mitochondrial genome is a closed circular DNA of 16,569 bp (1), which encodes for thirteen subunits of the oxidative phosphorylation complexes and for two rRNAs and 22 tRNAs of the mitochondrial protein synthesizing system (2). Deletions of mitochondrial DNA (mtDNA) have been reported in various human diseases, *i.e.*, mitochondrial myopathy (3), Kearns-Sayre syndrome (4,5), familial chronic progressive external ophthalmoplegia (6), encephalomyopathy (7), and Pearson's marrow/pancreas syndrome (8,9). The mechanism of mtDNA deletion and its relation to the etiology are largely unknown. Mapping of the deletions in a number of patients (3,5,6) revealed apparently identical deletions in several patients, suggesting nonrandom occurrence of deletions. There may be "hot spots" on the mtDNA that are more prone to deletion than other regions of mtDNA. To localize the hot spots precisely, we developed a method, which utilizes the combination of the polymerase chain reaction (PCR) and the S1 nuclease analysis (10). This PCR plus S1 method enabled us to choose primers for sequencing of the boundaries of the deletions in the present study.

In order to elucidate the molecular genetic basis of the hot spots of human mtDNA deletion, we have applied the asymmetric PCR amplification method (11) to direct sequencing of the PCR-amplified mtDNA from patients with myopathy.

0006-291X/89 $1.50

PATIENTS AND METHODS

Patients Five patients, Patient 1: a 21-year-old male, Patient 2: a 17-year-old female, Patient 3: a 32-year-old female, Patient 4: a 39-year-old female, and Patient 5: a 42-year-old female, had external ophthalmoplegia but had no pigmentary retinopathy. The Southern blot analysis of mtDNA from Patient 3 was reported previously (6).

Preparation of DNA The biopsied muscles (5 mg) were homogenized using a Physcotron Handy Micro Homogenizer (Niti-on, Tokyo) for 30 sec, and was digested in 1 ml of 10 mM Tris-HCl, 0.1 M EDTA (pH 7.4) containing 0.1 mg/ml proteinase K and 0.5% SDS. DNA was extracted twice with equal volumes of phenol/chloroform/isoamyl alcohol (25:25:1), once with chloroform/isoamyl alcohol (25:1). DNA was precipitated with a one-fiftieth volume of 5 M NaCl and two volumes of ethanol at -80°C for 2 h, and rinsed with 70% ethanol. The precipitated DNA was recovered in 30 μl of 10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0).

Synthesis of primers Primers for PCR were synthesized using a Shimadzu model NS-1 DNA synthesizer and an Applied Biosystems model 380B DNA synthesizer and purified on NENSORB Cartridges from Du Pont-NEN. The base sequences of the oligonucleotides are shown in Table 1.

Primary PCR amplification PCR amplification was carried out on 1 μl of the DNA solution (ca. 10 ng of total DNA) in a final volume of 100 μl which included 200 μM of each dNTP, 2.5 units of *Taq* DNA polymerase (Ampli*Taq*, Cetus) and PCR buffer (50 mM Tris-HCl, pH 8.3, containing 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin) with 1 μM each of primers (12). The combination of primers used for each patient is shown in Table 2. The reactions were carried out for a total of 35 cycles, with the use of a Perkin-Elmer/Cetus Thermal Cycler. The cycle time were as follows: denaturation 15 sec at 94°C; annealing, 15 sec at 45°C; and primer extension, 80 sec at 72°C.

PCR plus S1 analysis Fragments amplified from the deleted mtDNA and from the normal-sized mtDNA were mixed and subjected to heteroduplex formation. The deleted region of the mutant mtDNA was analyzed by the S1 nuclease digestion of the heteroduplexes according to the method reported previously (10).

Asymmetric PCR amplification PCR reamplification was carried out on 2.5 μl of the primary PCR product in a final volume of 100 μl which included the reagents described above with 0.01 μM of one primer and 1 μM of another primer essentially according to the method of

Table 1. Synthesized primers used for PCR

Primers*	Sequence 5'→3'	Complementary site**
L790	TGAACCTACGAGTACACCGA	7,901 to 7,920
L820	TTCATGCCCATCGTCCTAGA	8,201 to 8,220
L853	ACGAAAATCTGTTCGCTTCA	8,531 to 8,550
L909	ACACTTATCATCTTCACAAT	9,091 to 9,110
H1338	AACCTTAACAATGAACAAGA	13,400 to 13,381
H1363	CAGGTCAACCTCGCTTCCCC	13,650 to 13,631
H1479	CCACTCATTCATCGACCTCC	14,810 to 14,791
H1619	ACTTGCTTGTAAGCATGGG	16,219 to 16,191

*Primers L790, L820, L853, and L909 were used for amplification of the light strand of mtDNA. Primers H1338, H1363, H1479, and H1619 were used for amplification of the heavy strand of mtDNA.
**Numbering of mtDNA is according to Anderson et al. (1).

Table 2. Combinations of primers used for primary and asymmetric amplification of mtDNA and for sequencing of the templates

Patient	Primers for amplification	Primer for sequencing
1	L820 (0.01) + H1619 (1)	L853
2, 3, 4	L790 (1) + H1479 (0.01)	H1363
5	L820 (0.01) + H1338 (1)	L909

Figures in parentheses are concentration of primers in μM used for asymmetric amplification.

Gyllensten and Erlich (11). The combination of primers used for each patient is shown in Table 2. PCR was performed for a total of 35 cycles as above. The PCR product containing single-stranded DNA was precipitated with 0.6 volume of 20 % polyethylene glycol 6000 (PEG) containing 2.5 M NaCl essentially according to the method of Perbel (13). After incubation at 4°C for 1 h, the mixture was centrifuged at 11,000 × *g* for 10 min. The precipitate was suspended in 100 μl of 7.5% PEG containing 0.94 M NaCl and centrifuged as above. The pellet was rinsed with 0.5 ml of 70% ethanol, dried in vacuum for 15 min, and dissolved in 10 μl of distilled water. The removal of dNTP and primers by this PEG precipitation step is essential for reduction of the background in the subsequent sequencing.

DNA sequencing DNA was sequenced by the Sanger's dideoxynucleotide chain termination method (14) using the incorporation of α-[^{32}P]dCTP as the radiolabeling extension method. Sequenase reactions were performed using a kit supplied by United States Biochemicals. For the labeling reaction, the amplified single-stranded DNA-enriched template (7.5 μl, 0.5-1.0 pmol) was mixed with 1 μl of 10 μM sequencing primer listed in Table 2, and the primer-template mixture was heated to 100°C for 10 min and immediately placed on ice. Reactions were initiated by adding 8.8 μl of this mixture to 5.2 μl of dideoxy G, A, T, and C reaction mixtures composed of reagents provided in the "Sequence" kit as follows: 2 μl of five-times diluted "labeling mix", 0.25 μl of Sequenase (3.25 units), 1.75 μl of "dilution buffer", 2.2 μl of "5× Sequenase buffer", and 1 μl of 0.1 M DTT. The mixture was incubated at 37°C for 2 min. The product (3.5 μl) was transferred to four tubes containing 2.5 μl of one of four "termination mixes" and incubated at 37°C for 2 min. After addition of 4 μl of "stop solution", the mixture was heated to 100°C for 3 min and 3 μl was loaded onto a 6% polyacrylamide/7M urea sequencing gel.

RESULTS

The region surrounding the mtDNA deletion of Patient 1 was amplified by the PCR method using primers L820 and H1619, the distance between which was 8.0 kb. A single band of 1.0 kb was amplified consistently with a 7.0-kb deletion. The deletion extended at least from position 9,201 to 16,209. The deleted region of uncertainty was further narrowed by the PCR plus S1 analysis. The location of the deletion was calculated to be from position 8,650 ± 50 to position 15,590 ± 60. According to the result of the PCR plus S1 analysis, sequencing primer L853 was chosen for the direct sequencing of the deleted mtDNA. The single-strand templates were prepared by using the asymmetric PCR method, and were sequenced using Sanger's dideoxynucleotide termination method without cloning. The crossover sequence was found to be a directly repeated sequence containing four cytosines (5'-

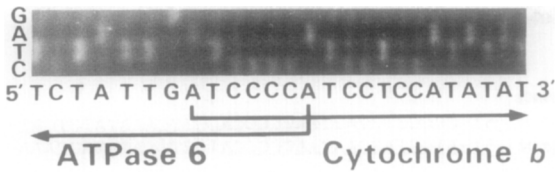

Fig. 1. PCR amplification and direct sequencing of the deleted mtDNA from the skeletal muscle of Patient 1. Shown is a portion of an autoradiograph of sequencing gel of the amplified DNA from the deleted mtDNA.

ATCCCCA-3'), flanked by AT-rich regions (Fig. 1). The directly repeated sequence was located in the boundaries of the deletion between the ATPase 6 gene and the cytochrome *b* gene. When the wild-type sequences of the ATPase 6 and the cytochrome *b* genes were compared each other without adding a gap, almost no sequence homologies were obtained except in the crossover sequence. But when they were compared with addition of a gap, additional homologies were found in 11 of 12 bases adjacent to the 3'-side of the directly repeated sequence. Therefore, the deletion spanned 7,039 bp, probably starting from position 8,624 at the 3'-side of the directly repeated sequence within the ATPase 6 gene, and ends at position 15,662 at the 3'-end of the directly repeated sequence within the cytochrome *b* gene (Fig. 2).

In Patient 2, the deleted mtDNA was amplified by the PCR method using primers L790 and H1479, the distance between which was 6.9 kb. A single band of 1.9 kb was amplified consistently with a 5.0-kb deletion. Direct sequencing using primer H1363 revealed that the crossover sequence was found to be a 13-bp directly repeated sequence of 5'-ACCTCCC TCACCA-3' (Fig. 2), which was located in the boundaries of the deletion between the ATPase 8 gene and the ND5 gene. The deletion spanned 4,977 bp from position 8,483 to position 13,459. The directly repeated sequence was flanked by AT-rich regions. When the wild-type sequences of the ATPase 8 and the ND5 genes were compared each other, no significant homologies were found in the region surrounding the directly repeated sequence.

In Patient 3, the Southern blot analysis showed apparently a single mtDNA deletion (6), but the PCR method detected multiple mtDNA deletions (data not shown). In Patient 4, multiple deletions, namely multi-types of mutated mtDNAs, were also detected by the PCR method. We selectively amplified a mutated mtDNA of relatively large population in Patients 3 and 4 with the same primers used for Patient 2. Direct sequencing of the PCR product demonstrated that the deletion was identical with the 4, 977-bp deletion found in Patient 2.

In Patient 5, the deleted mtDNA was amplified by the PCR method using primers L820 and H1338, the distance between which was 5.2 kb. A single band of 1.5 kb was amplified consistently with a 3.7-kb deletion. Direct sequencing using primer L909 revealed that the crossover sequence was a 3-bp directly repeated sequence of 5'-CCT-3' (Fig. 2), which was located in the boundaries of the deletion between the ATPase 6 gene and the ND5 gene. The deletion spanned 3,717 bp from position 9,192 to position 12,908. When the wild-type sequences of the ATPase 6 and the ND5 genes were compared each other, homologies were found in the regions adjacent to the directly repeated sequence.

Type 1 deletion Patient 1: 7,039-bp deletion

```
                                        8,624
Wild-type sequence                      ↓
ATPase 6 gene       5'-CCCCCTCTATTGATCCCCACCTCCAAATATCTCAT-3'
Cytochrome b gene   5'-ATCCTAGCAATAATCCCCATCCTCCATATACCAAA-3'
                                        ↑
                                        15,662
Deleted mtDNA sequence
hybrid gene         5'-CCC CCT CTA TTG ATC CCC ATC CTC CAT ATA TCC AAA-3'
hybrid protein        -Pro Pro Leu Leu Ile Pro Ile Leu His Met Ser Lys-
```

Type 2 deletion Patients 2, 3, and 4: 4,977-bp deletion

```
                                        8,483
Wild-type sequence                      ↓
ATPase 8 gene       5'-AACTACCACCTACCTCCCTCACCAAAAGCCCATAAA-3'
ND5 gene            5'-CTCTCACTTCAACCTCCCTCACCATTGGCAGCCTAG-3'
                                        ↑
                                        13,459
Deleted mtDNA sequence
hybrid gene         5'-AAC TAC CAC CTA CCT CCC TCA CCA TTG GCA GCC TAG-3'
hybrid protein        -Asn Tyr His Leu Pro Pro Ser Pro Leu Ala Ala stop
```

Type 3 deletion Patient 5: 3,717-bp deletion

```
                                        9,192
Wild-type sequence                      ↓
ATPase 6 gene       5'-CTTCTAGTAAGCCTCTACCTGCACGACAACACATAA-3'
ND5 gene            5'-GCCTTAGCATGATTTATCCTACACTCCAACTCATGA-3'
                                        ↑
                                        12,908
Deleted mtDNA sequence
hybrid gene         5'-CTT CTA GTA AGC CTC TAC CTA CAC TCC AAC TCA TGA-3'
hybrid protein        -Leu Leu Val Ser Leu Tyr Leu His Ser Asn Ser Trp-
```

Fig. 2. Comparison of the nucleotide sequences of the wild-type genes with those of the hybrid genes in the deleted mtDNAs from Patients 1-5. The sequence of the wild-type genes are shown above the sequence of the hybrid gene resulted from deletion of mtDNA in each patient. Bold letters indicate the directly repeated sequences. Underlined is the excised sequence in mtDNA deletion in each patient. Numerals indicate the nucleotide numbers (1).

DISCUSSION

The present results have demonstrated that three different types of mtDNA deletions occur between the directly repeated sequences present in the wild-type mtDNA (Figs. 1 and 2). The first type of deletion, a 7,038-bp deletion between the ATPase 6 gene and the cytochrome *b* gene, was found only in Patient 1 out of 8 patients with large deletions studied in our laboratory. We found the 7-bp directly repeated sequence, 5'-ATCCCCA-3', in the boundaries of this deletion (Fig. 2). In contrast, the second type of deletion, a 4,977-bp deletion between the ATPase 8 gene and the ND5 gene, was found in Patients 2, 3, and 4. The

13-bp directly repeated sequence, 5'-ACCTCCCTCACCA-3' was found in the boundaries of this deletion (Fig. 2). The same deletion was reported in a patient with Pearson's marrow/pancreas syndrome by Rotig et al. (9) and in 19 out of 29 patients with Kearns-Sayre syndrome and progressive external ophthalmoplegia by Schon et al. (15). The third type of deletion, a 3,717-bp deletion between the ATPase 6 gene and the ND5 gene, was found only in Patient 5 in our laboratory. The 3-bp directly repeated sequence, 5'-CCT-3' was found in the boundaries of this deletion (Fig. 2). Because additional homologies were found adjacent to the directly repeated sequences in Patients 1 and 5, the deletions might be promoted by not only the short directly repeated sequences but also by the homologous sequences surrounding them.

As shown in Fig. 2, the sequence of the mutant mtDNA with the first type of deletion forms an open reading frame which predicts a 12-kDa hybrid protein composed of 32 amino acid residues from the N-terminal side of the ATPase subunit 6 and of 75 amino acid residues from the C-terminal side of the cytochrome *b* protein. The second type of deletion introduces a stop codon 12 nucleotides aside from the boundary, resulting in a premature termination of the ND5 protein. Therefore, the sequence of the mutant mtDNA predicts a 5-kDa abnormal protein composed of 42 amino acid residues from the N-terminal side of the ATPase subunit 8 and of 3 amino acid residues resulted from a frame shift in the ND5 gene. The sequence of the mutant mtDNA with the third type of deletion forms an open reading frame which predicts a 69-kDa hybrid protein composed of 222 amino acid residues from the N-terminal side of the ATPase subunit 6 and of 412 amino acid residues from the C-terminal side of the ND5 protein. Because the ATPase subunits 6 and 8 in Complex V, the ND5 protein in Complex I, and the cytochrome *b* protein in Complex III play central roles in energy transducing function of each complex, it should be examined whether the message for these hybrid proteins could be translated into proteins and whether they could disturb the molecular assembly of these energy-transducing complexes or not.

Human mtDNA is very small and economically packed; the rRNA, tRNA, and the protein-coding genes are organized in the mtDNA continuously with no non-coding sequences, except for the D-loop region involved in the regulation of the replication and translation of the mtDNA (16). Therefore, any mutation in the mtDNA will involve a functionally important part of the genome. In the present study, three types of directly repeated sequences responsible for the deletions were found to be located within the protein-coding genes. In contrast with the recombination of nuclear genes which occurs mainly between non-coding regions (17), recombination of mtDNA will occur inevitably between the protein-coding genes. The recombinational events in mtDNA, which we could designate as pseudo-recombination, might result in formation of hybrid genes and thereby affect the mitochondrial bioenergetic function.

We have recently described multiple deletions of mtDNA in a patient with familial ocular myopathy (12). In the present paper, we found multiple mtDNA deletions in Patients 3 and 4. The boundary sequence of one of the deleted mtDNA in these patients was identical with that of the second type of deletion. Therefore, it is likely that directly repeated sequences are involved not only in the single mtDNA deletion but also in the multiple mtDNA deletions observed in patients with myopathy. Zeviani et al. described multiple mtDNA deletions

starting exclusively at the D-loop region in a family with autosomal dominant mitochondrial myopathy (18). In contrast, we found multiple mtDNA deletions outside the D-loop region both in the present patients and in the previous patient with familial ocular myopathy (12). Therefore, the mechanism responsible for the multiple mtDNA deletions described here apparently differs from the mechanism proposed by Zeviani et al. for the multiple mtDNA deletions in their cases (18).

We have proposed a hypothesis that accumulation of somatic mitochondrial gene mutations will eventually lead to accumulation of partially or grossly bioenergetic defective cells with time as a key facet of the processes of ageing and degenerative diseases (19). The directly repeated sequences at the boundaries of deletions reported here might contribute to the hypothesized accumulation of mtDNA deletions associated with these processes. Since directly repeated sequences of relatively short base pairs, similar to the sequences reported here, are abundant in mtDNA, these sequences could provide numerous chances for mtDNA mutations.

ACKNOWLEDGMENTS

We thank Assoc. Prof. Ko Sahashi and Dr. Toru Ibi, Department of Medicine, Division of Neurology, Aichi Medical University, Dr. Ikuya Nonaka, Division of Ultrastructural Research, National Institute of Neuroscience, Prof. Akira Takahashi, Department of Neurology, Faculty of Medicine, University of Nagoya, and Prof. Yoshihiro Wakayama, Department of Neurology, Fujigaoka Hospital, Showa University School of Medicine for permission to study the patients' muscles. Oligonucleotides were synthesized at the Laboratory of Biomolecular Analysis, Faculty of Medicine and at the Center for Gene Research, University of Nagoya. This work was supported in part by the Grants-in-Aid for General Scientific Research (62570128) to M.T., and for Scientific Research on Priority Areas (Bioenergetics, 01617002) to T.O., from the Ministry of Education, Science, and Culture of Japan and by Grant 01-02-39 from the National Center for Nervous, Mental and Muscular Disorders of the Ministry of Health and Welfare, Japan, to T.O.

REFERENCES

1. Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R., and Young, I.G. (1981) Nature 290, 457-465.
2. Chomyn, A., Mariottini, P., Cleeter, M.W.J., Ragan, C.I., Matsuno-Yagi, A., Hatefi, Y., Doolittle, R.F., and Attardi, G. (1985) Nature 314, 592-597.
3. Holt, I.J., Harding, A.E., and Morgan-Hughes, J.A. (1988) Nature 331, 717-19.
4. Lestienne, P., and Ponsot, G. (1988) Lancet i, 885.
5. Zeviani, M., Moraes, C.T., DiMauro, S., Nakase, H., Bonilla, E., Schon, E.A., and Rowland, L.P. (1988) Neurology 38, 1339-1346.
6. Ozawa, T., Yoneda, M., Tanaka, M., Ohno, K., Sato, W., Suzuki, H., Nishikimi, M., Yamamoto, M., Nonaka, I., and Horai, S. (1988) Biochem. Biophys. Res. Commun. 154, 1240-1247.
7. Noer, A.S., Marzuki, S., Trounce, I., and Byrne, E. (1988) Lancet ii, 1253-1254.
8. Rotig, A., Colonna, M., Blanche, S., S., Fischer, A., Le Deist, F., Frezal, J., Saudubray, J.-M., and Munnich, A. (1988) Lancet ii, 567-568.
9. Rotig, A., Colonna, M., Bonnefont, J.P., Blanche, S., Fischer, A., Saudubray, J.M., and Munnich, A. (1989) Lancet i, 902-903.
10. Tanaka-Yamamoto, T., Tanaka, M., Ohno, K., Sato, W., Horai, S., and Ozawa, T. (1989) Biochim. Biophys. Acta (in press).
11. Gyllensten, U.B., and Erlich, H.A. (1988) Proc. Natl. Acad. Sci. USA 85, 7652-7656.
12. Sato, W., Tanaka, M., Ohno, K., Yamamoto, T., Takada, G., and Ozawa, T. (1989) Biochem. Biophys. Res. Commun. (in press).

13. Perbel, B. (1988) Practical Guide to Molecular Cloning, pp. 636-640. John Wiley & Sons, New York.
14. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
15. Schon, E.A., Rizzuto, R., Moraes, C.T., Nakase, H., Zeviani, M., and DiMauro, S. (1989) Science 244, 346-349.
16. Attardi, G. (1981) Trends Biochem. Sci. 89, 100-103.
17. Vnencak-Jones, C.L., Phillips, J.A.III, Chen, E.Y., and Seeburg, P.H. (1988) Proc. Natl. Acad. Sci. USA 85, 5615-5619.
18. Zeviani, M., Servidei, S., Gellera, C., Bertini, E., DiMauro, S., and DiDonato, S. (1989) Nature 339, 309-311.
19. Linnane, A.W., Marzuki, S., Ozawa, T., and Tanaka, M. (1989) Lancet i, 642-645.