Different mechanisms inferred from sequences of human mitochondrial DNA deletions in ocular myopathies

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ABSTRACT

We have sequenced the deletion borders of the muscle mitochondrial DNA from 24 patients with heteroplasmic deletions. The length of these deletions varies from 2.310 bp to 8.476 bp and spans from position 5.786 to 15.925 of the human mitochondrial genome preserving the heavy chain and light chain origins of replication.

12 cases are common deletions identical to the mutation already described by other workers and characterized by 13 bp repeats at the deletion boundaries, one of these repeats being retained during the deletion process. The other cases (10 out of 12) have shown deletions which have not been previously described. All these deletions are located in the H strand DNA region which is potentially single stranded during mitochondrial DNA replication.

In two cases, the retained Adenosine from repeat closed to the heavy strand origin of replication would indicate slippage mispairing. Furthermore in one patient two mt DNA molecules have been cloned and their sequences showed the difference of four nucleotides in the breakpoint of the deletion, possibly dued to slippage mispairing.

Taken together our results suggest that deletions occur either by slippage mispairing or by internal recombination at the direct repeat level. They also suggest that different mechanisms account for the deletions since similarly located deletions may display different motives at the boundaries including the absence of any direct repeat.

INTRODUCTION

Mitochondrial DNA reorganization, without any apparent deleterious effects, has long been documented in eukaryotes including yeast and fungi (for a review see 1), plants (2), Drosophila (3), lizards (4) and mice (5).

However, the organization of the human circular mitochondrial DNA (mt DNA) of 16.569 bp (6) has been studied in mitochondrial diseases. Deletions of the mt DNA have been reported recently in myopathies (7) and neuromuscular diseases such as Kearns-Sayre syndrome (8,9) and chronic external ophthalmoplegia (10). Although these deletions differ in length and position between individuals, they are similar within different tissues of the same patient (11,12,13) suggesting early clonal events in the pleiotropic dispersion of these deleted genomes. It is notable that the normal mtDNA molecule is also present in all tissues providing general heteroplasmy. Passive or active mechanisms occurring spontaneously during embryogenesis may be responsible for the formation of the deletions which in one case have been reported to be a dominant trait without maternal transmission of the deleted genome (14).

In order to detect any common mechanism for the deletion process, the deletion boundaries of mutated mitochondrial DNA from 24 patients have been sequenced. In most of the cases direct repeats ranging from 4 to 13 bp were detected near the breakpoint following sequence comparison with the normal molecule, suggesting that recombination has occured. However no general rule is applicable since in 2 cases no direct repeat was found at deletion boundaries.

MATERIALS AND METHODS

Mapping of deleted mitochondrial DNA

Preparation of muscle DNAs and mapping of the heteroplasmic deletions in mitochondrial DNA (mtDNA) in each patient have been previously described (11).

PCR and DNA sequencing

Based on mapping data (11), the mt DNA area encompassing a deletion was amplified by the PCR method using oligonucleotide primers corresponding to mtDNA sequences located immediately upstream and downstream of each breakpoint (11). In five cases,

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the PCR product was cloned in M13 and single stranded DNA was sequenced using sequenase (USB).In some cases direct sequencing was performed after direct asymmetric amplification (primers in a ratio of 1 pmole to 50 pmoles), in the other cases the double stranded PCR product was subjected to asymmetric amplification using primers in a ratio of 0.3 to 30 pmoles. Single-strand sequencing was performed with forward primer using dideoxynucleotide termination (Sequenase kit (USB) or T7 Polymerase Kit (Pharmacia)).

Computer analysis

The sequence searches and comparison have been made with the help of Microgenie (Beckman) and using Anderson's sequence as a reference (6).

RESULTS

We have investigated mtDNA deletions from 24 patients. The results shown on table I display new breakpoint sequences compared to those already described (16,17,18).

Table I. Classification of the different deletions mapped at the nucleotidic level in patients presenting Kearns-Sayre syndrome or progressive external ophthalmoplegia.

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I   1 (deletion size : 3.512 bp)
                7491                                11003
    TGGTTTCAAGCCAAC(CCCATGGCCTC......TGGCAAGCCAAC)GCCACTTATCCAG
    tRNASer                                                ND4
    2-11 COMMON DELETION (deletion size : 4.977 bp)(10 cases)*
          8470          8482              13447          13459
    TACCACCTACCTCCCTCACCA(AAGCCCA....TTCAACCTCCCTCACCA)TTGGCAGCCTA
    ATPase8                                                ND5
    12 (deletion size : 5.261 bp)
                  8624                                13885
    CTATTGATCCCCAC(CTCCAAATATC.....TAAAATAAAATCCCCAC)TATGCACATTTTA
    ATPase6                                                ND5
    13 (deletion size : 7.031 bp)
                    8823                          15854
    ACCAACCACCCAACTATCT(ATAAACCTAG...TACCAACTATCT)CCCTAATTGAAAAC
    ATPase6                                            Cytb
    14 (deletion size : 7.767 bp)*
                   7669                           15436
    TTTCATGATCACGCCCTC(ATAATCA.....TCAAAGACGCCCTC)GGCTTACTTCTCTTC
    CoxII                                              Cytb
II  15 (deletion size : 2.310 bp)
              12103                          14413
    ATTCTCCTCCTATCC(CTCAACCCCGACAT.....CAAGACC)TCAACCCCTGACCCCCA
    ND4                                                ND6
    16 (deletion size : 4.265 bp)
             10169                        14434
    AATCCACCCCTTAC(GAGTGCGGC....ACCCCCATGCC)TCAGGATACTCCTCAATAGCCA
    ND3                                                ND6
    17 (deletion size : 4.417 bp)
             11368                          15785
    ACACAATAGCTTTT(ATAGTAAAGA.....TAAGCTACCCTT)TTACCATCATTGG
    ND4                                            Cytb
    18 and 19 (deletion size : 4.977 bp)(2 cases)
       8468                          13445
    TACCACC(TACCTCCCTCACCAAAGC....CACTTC)AACCTCCCTCACCATTGGCAGCCTA
    ATPase8                                            ND5
    20 (deletion size : 5.100 bp)
              9180                          14280
    TCACACTTCTAGTA(AGCCTCTA.....TGAACCCTGA)CCCCTCTCCTTCATAAATTA
    ATPase6                                            ND6
    21 (deletion size : 6.032 bp)
             8563                           14595
    TTCATTGCCCCCA(CAATCCTAGGCC.....AAACCCCCATA)AATAGGAGAAGGCTTAG
    ATPase6
    ATPase8                                            ND6
    22 (deletion size : 8.136 bp and 8.132 bp)*
          5786                             13922
    GAAGCTGCTT(CTTCGAATTTGCAAT......CTCGGATTCTA)CCCTAGCATCACCAC
    tRNACys                                            ND5
          5787                            13919
    GAAGCTGCTTC(TTCGAATTTGCAAT......CTCGGATT)CTACCCTAGCATCACCAC
    tRNACys                                            ND5
III 23 (deletion size : 4.665 bp)*
                8571                          13236
    TGCCCCCACAATCCTA(GGCCTACCCG.....GACATCAAAAAA)ATCGTAGCCTTCTCC
    ATPase6                                            ND5
    ATPase8
    24 (deletion size : 8.476 bp)*
                  7449                        15925
    CATAAAATCTAGACAAA(AAAGGAAG......CTTGTAAAC)CGGAGATGAAA
    CoxI          tRNASer                          tRNAThr
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* indicates cloned amplification products.

Class I refers to perfect direct repeats at the deletion boundaries, class II corresponds to imperfect repeats at deletion boundaries, class III no repeats present. Deletion boundaries of patient 23 has been previously reported in reference 15. Note the 2 sequences obtained after M13 cloning of mtDNA from patient 22.

22 out of 24 deletions present direct repeats at their boundaries. The left borders mapped between nucleotides 5.786 (patient 22) to 12.103 (patient 15) and right borders from 11.004 (patient 1) to 15.926 (patient 24). The length of the deletions varies from 2.310 bp (patient 15) to 8.476 (patient 24), more than the half of the human mitochondrial genome.

The distribution of the breakpoints occurs randomly along the 10 Kb fragment between the light and the heavy strand origins of replication (figure 1). However in 12 cases (patients 2 to 11

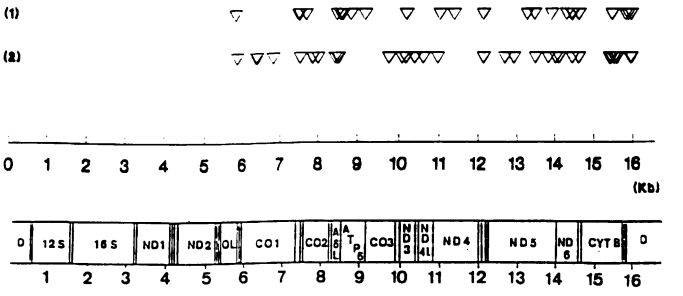

Figure 1. Repartition of deletion breakpoints in the mt DNA . Each breakpoint is indicated by a triangle.
(1) indicates the data of Mita et al.,1990.
(2) shows our data.

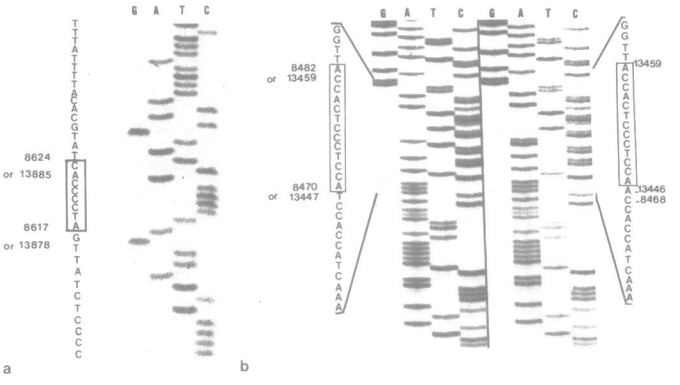

Figure 2. Autoradiogram showing the breakpoint in 3 cases: a- the sequence of deletion boundaries in patient 12 (8 bp repeat). b-comparison of the common deletion sequence (left panel) and the supplementary Adenosine at position 13.446 (right panel).

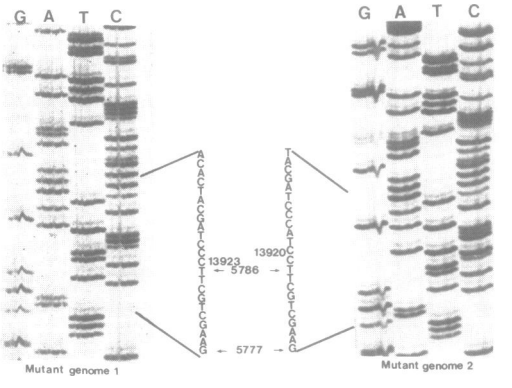

Figure 3. Autoradiogram showing the two mitochondrial genomes of patient 22 with differences in breakpoint sequences.

and patients 18 and 19) an identical sized deletion of 4.977 bp was observed. There is a 13 bp repeat in the normal molecule at the defined edges of this deletion, one of the repeats being retained during the deletion process. In 10 of these cases (patients 2 to 11) it is unsure whether the direct repeat located near the heavy strand origin of replication or that located near the light strand origin is retained during the deletion process. However in 2 of these 12 cases (cases 18 and 19) there is an additional Adenosine which flanks the first direct repeat and which is found at the deletion boundaries. Thus, in these two cases, the first direct repeat is conserved during the deletion process in agreement with the slippage mispairing mechanism as suggested in reference 18. The sequence comparison of one of theses cases is shown on figure 2. Deletions showing perfect direct repeats, which can be allocated to class I as suggested in reference 16 were present in 14/24 (58%) of the cases (patients 1 to 14). 8 (33%) cases can be defined as class II with the direct repeat located within or at the proximity of the breakpoint (patients 15 to 22). The last 2 deletions fall into class III without any evident feature.

Close to the light strand origin of replication, the mtDNA from patient 22 (class II) presents 2 identical deletions in two clones (the upper in table 1) and one different in one clone at the $tRNA^{Cyst}$ level with a 4 bp repeat and left slippage of a Cytosine but retention of one Cytosine, one Thymine and one Adenine at the right border, so that both molecules differ by 4 nucleotides in length as shown on figure 3.

Deletions borders locating in tRNA genes are found in 5 breakpoint sequences. Interestingly the longest deletion reported so far of 8.476 bp is associated with tRNA genes of the light strand ($tRNA^{Ser}$) and of the heavy strand ($tRNA^{Thr}$) in patient 24. The left deletion borders are more frequently associated in 16 cases with the short ATPase 6 and 8 genes, from position 8.468 to 8.823, as well as NADH Dehydrogenase subunit 5 at the right border from position 13.237 to 13.923 in 15 cases.

DISCUSSION

The main conclusion of these studies describing mtDNA deletions in human pathologies is the confirmation of the importance of base pair repeats at the deletion boundaries, one of these repeats being eliminated during the deletion process in agreement with previous findings (16−21). This may suggest specific site recombination (for a review see 22,23) of the mtDNA molecule involving recombinases such as those isolated from Drosophila Melanogaster embryos (24), FLP recombinase of yeast (25,26), CRE bacteriophage P1 (27), Rec A protein (28) or present in human nuclei (29).

Another proposed mechanism in favor for the deletions of the mtDNA is slippage mispairing as suggested by Shoffner et al. in reference 18. This process could explain the deletions for at least 3 patients, two presenting the common deletion but with retention of an Adenosine and patient 22 which displays two mt DNA sequences different by 4 nucleotides. In these cases, the first repeat close to the H strand origin of replication would be displaced during the H strand synthesis and would base pair with the second direct repeat during the branch migration of the replication complex towards the light strand origin of replication. Degradation of the heavy displaced strand could be mediated by a single strand DNase as reported for the mouse (30). Another mitochondrial endonuclease activity, specific for UV irradiated DNA, has been recently characterized in mammalian (31).

It may be significant that all but one deletion (7) map in the region where the heavy strand DNA is displaced during H strand replication (11) suggesting cleavage of this parental strand as proposed in the model described in reference 15.

The class of deletion where no base pair repeat is distinguishable (class III) may be explained by illegitimate recombination (for a review see 32) mediated by topoisomerases (33,34). Non-homologous DNA with protuberant single strand may be repaired by illegitimate recombination (35).

However search for homologies with cleavage sites for topoisomerases I and II revealed no such sites. In effect the transcription and the replication processes in the mammalian mitochondrial DNA are not symmetric and the heavy strand promoter is more active than the light strand. This may induce positive or negative supercoils which could be resolved with errors by topoisomerases and gyrases(36,37).

In conclusion the results reported here may provide an experimental model for studying human DNA reorganization. Presently there is no conclusive argument in favor of a somatic as opposed to a germinal deletion process. One report of maternal inheritance (38) show that the deletions differ in position between the mother and the patient. During the oocyte maturation the mitochondrial DNA may undergo 8 to 9 replication cycles (39). If we assume that only one to 2.000 DNA molecules undergoes a deletion of half of its size, then due to a simple kinetic effect, assuming that only half the time is required for the replication of the deleted molecule, at the end of the ovocyte maturation the deleted molecule may be as numerous as the normal one. During partition in the different stem cells, the deleted genomes may segregate randomly or preferentially in different tissues providing the various phenotype we observed in this new class of disease.

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REFERENCES

1. Dujon,B. and Belcourt,L. (1984) in: Mobile DNA, Berg,D. and Howe,M., Eds., American society for microbiology, 861−878.
2. Leaver,C.J., Isaac,P.G., Small,I.D., Bayley-serres,J., Limmell,A.P. and Hawkesford,M.J. (1988) Phil. Trans. R. Soc. Lond. 319, 165−176.
3. Solignac,M., Monnerot,M. and Mounolou,J.C. (1983) Proc.Natl.Acad.Sci. USA 80, 6942−6946.
4. Moritz,C. and Brown,W.M. (1986) Science 233, 1425−1427.
5. Boursot,P., Yonekawa,H. and Bonhomme,F. (1987) Mol. Biol. Evol. 4, 46−55.
6. Anderson,S., Bankier,A.T., Barrel,B.G., Debruijn,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.
7. Holt,I.J., Harding,A.E. and Morgan-Hughes, .J.A. (1988) Nature 331, 717−719.
8. Lestienne,P. and Ponsot,G. (1988) Lancet i, 885−885.
9. Zeviani,M., Moraes,C.T., DiMauro,S., Nakase,H., Bonilla,E., Schon, .E.A. and Rowland,L.P. (1988) Neurology 38, 1339−1346.
10. Moraes,C.T., DiMauro,S., Zeviani,M., Lombes,A., Shanske,S., Miranda,A.F., Nakase,H., Bonilla,E., Werneck,L.C., Servidei,S., Nonaka,I., Koga,Y., Spiro,A.J., Brownell,A.K.W., Schmidt,B., Schotland,D.L., Zupanc,M., DeVivo,D.C., Schon,E.A. and Rowland,L.P. (1989) N. Eng. J. Med. 320, 1284−1299.

11. Nelson,I., Degoul,F., Obermaier-Kusser,B., Romero,N., Marsac, C., Vayssiere,J.L., Gerbitz,K., Fardeau,M., Ponsot, G. and Lestienne,P. (1989) Nucl. Acid. Res. 17, 8117–8124.
12. Obermaier-Kusser,B., Muller-Hocker,J., Nelson,I., Lestienne,P., Enter,C., Riedele,T. and Gerbitz,K.D. (1990) Biochem. Biophys. Res. Commun. 169, 1007–1015.
13. Shanske,S., Moraes,C.T., Lombes,A., Miranda,A.F., Bonilla,E., Lewis,B., Whelan,M.A., Ellsworth,C.A. and DiMauro,S. (1990) Neurology 40, 24–28.
14. Zeviani,M., Servidei,S., Gerella,C., Bertini,E., DiMauro,S. and DiDonato,S. (1989) Nature 339, 309–311.
15. Nelson,I., d'Auriol,L., Galibert,F., Ponsot,G. and Lestienne,P. (1989.) C.R.Acad.Sci. 309, 403–407.
16. Mita,S., Rizzuto,R., Moraes,C.T., Shanske,S., Arnaudo,E., Fabrizi,G.M., Koga,Y., DiMauro,S. and Schon,E.A. (1990) Nucleic Acids Research 18, 561–567.
17. Holt,I.J., Harding,A.E. and Morgan-Hughes,J.A. (1989) Nucl. Acid Res. 17, 4465–4469.
18. Shoffner,M.T., Lott,A.S., Voljavec,S.A., Soueidan,D.A., Costigan,D.A. and Wallace,D.C. (1989) Proc. Nat. Acad. Sci. 86, 7952–7956.
19. Schon,E.A., Rizzuto,R., Moraes,C.T., Nakase,H., Zeviani,M. and DiMauro,S. (1989) Science 244, 346–349.
20. Rotig,A., Colonna,M., Blanche,S., Fisher,A., Ledeist,F., Frezal,J., Saudubray,J.M. and Munnich,A. (1988) The Lancet i, 567–568.
21. Johns,D.R., Rutledge,S.L., Stine,O.C. and Hurko,O. (1989) Proc. Natl. Acad. Sci. 86, 8059–8062.
22. Sadowski,P. (1986) J. Bacteriol. 165, 341–347.
23. Gellert,M. and Nash,H. (1987) Nature 325, 401–404.
24. Eisen,A. and Camerini-Otero,R.D. (1988) Proc. Natl. Acad. Sci. USA 85, 7481–7485.
25. Golic,K.G. and Lindquist,S. (1989) Cell 59, 499–509.
26. Meyer-Leon,L., Gates,C.A., Attwood,J.M., Wood,E.A. and Cox,M.M. (1987) Nucl. Acid Res. 15, 6469–6489.
27. Hoess,R., Wierzbicki,A. and Abremski,K. (1987) Proc. Natl. Acad. Sci. USA 84, 6840–6844.
28. Radding,C.M. (1982) Ann. Rev. Genet. 16, 405–437.
29. Lopez,B. and Coppey,J. (1989) Biochem. Biophys. Res. Commun. 158, 454–461.
30. Tomkinson,A.E. and Linn,S. (1986) Nucl. Acid Res. 14, 9579–9593.
31. Tomkinson,A.E., Bonk,R.T., Kim,J., Bartfeld,N. and Linn,S. (1989) Nucl. Acid Res. 18, 929–935.
32. Ehrlich,S.D. (1989) I, in: Mobile DNA, Berg,D. and Howe,M., Eds., American Society for Microbiology,799–832.
33. Liu,L.F., Rowe,T.C., Yang,L., Tewey,K.T. and Chen,G.L. (1983) J. Biol. Chem. 258, 15365–15370.
34. Bae,Y., Kawasaki,I., Ikeda,H. and Liu,L.F. (1988) Proc. Natl. Acad. Sci. 85, 2076–2080.
35. Thode,S., Schafer,A., Pfeiffer,P. and Vielmetter,A. (1990) Cell 60, 921–928.
36. Frank-kamenetskii,M. (1989) Nature 337, 206–206.
37. Wu,H.Y., Shyy,S., Wang,J.C. and Liu,L.F. (1988) Cell 53, 433–440.
38. Ozawa,T., Yoneda,M., Tanaka,M., Ohmo,K., Sato,W., Suzuki,H., Nishikimi,M., Yamamoto,M., Nonaka,I. and Horai,S. (1988) Biochem. Biophysic. Res. Comm. 154, 1240–1247.
39. Hauswirth,W.W. and Laipis,P.J. (1985) Achievement and perspectives in mitochondrial research , vol II, ed. Quagnariello Elsevier US Amsterdam .