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Mapping of heteroplasmic mitochondrial DNA deletions in Kearns–Sayre syndrome

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ABSTRACT

Kearns–Sayre syndrome (KSS) is a progressive neuromuscular disease characterised by ophtalmoplegia, cardiac bloc branch, pigmentary retinopathy associated with abnormal mitochondrial function. We have studied the mitochondrial DNA organization of patients presenting KSS and have found large deletions ranging from 3 to 8,5 kilobase pairs. DNA molecules containing deletion are accompanied by the presence of the normal sized mtDNA molecule forming heteroplasmic genomes. The deletions always map in the region which is potentially single stranded during mitochondrial DNA replication. The deletions differ in lengh and position between individuals but are similar within the different tissues of an individual suggesting that they arise during or before embryogenesis.

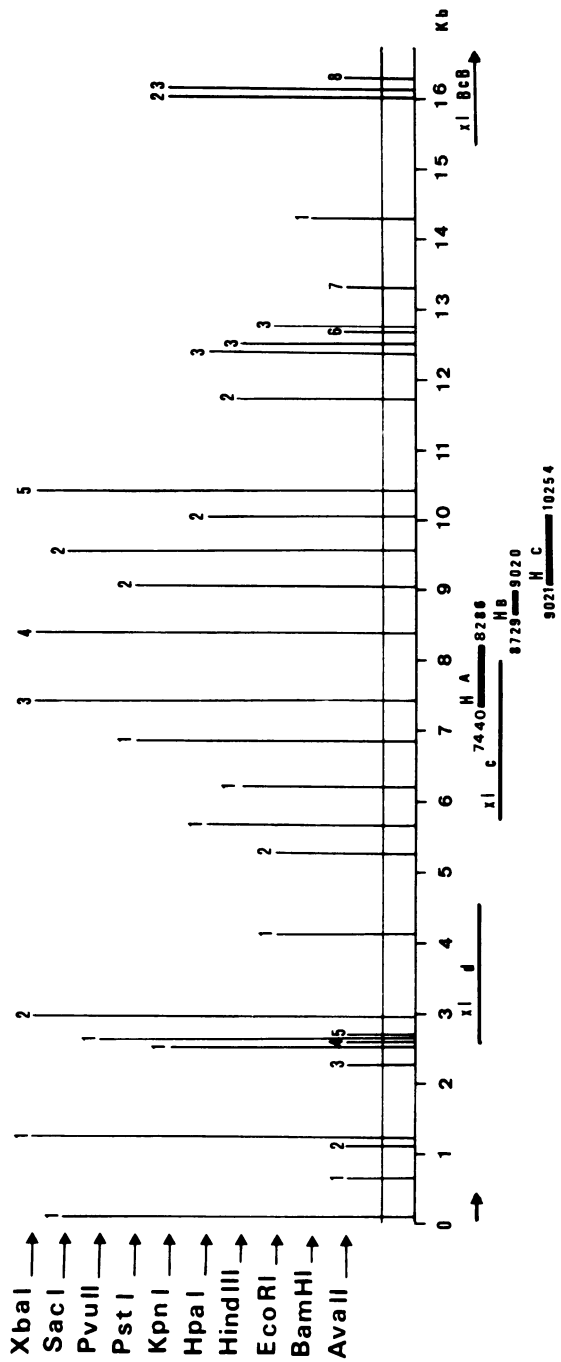
INTRODUCTION

Kearns–Sayre syndrome (KSS) is a rare but progressive and seriously debilitating disease. The age of onset is generally below 20 and the degree of severity differs between patients as well as the pronostic which may be fatal. KSS is characterized by a pigmentary retinopathy, ophtalmoplegia and cardiac conduction defects. The disease is associated with increased protein level in the cerebrospinal fluid, and pathological indices including muscle 'Ragged Red Fibers' upon trichrome gomori staining (1). Enzymatic defects at the respiratory chain complexes I, III, IV and V have been associated with KSS as well as abnormal protein synthesis in mitochondria (2). Treatment with coenzyme Q_{10} generally yields an improvement of the state of the patients.

The mitochondrial genome is a closed circular DNA molecule of 16,569 base pairs containing the specific genetic code for the 13 protein subunits of the respiratory chain complexes I, III, IV and V, 2 ribosomal RNA molecules and 22 transfer RNA molecules (3). Deletions of muscle mitochondrial DNA have been found in patients presenting with mitochondrial myopathies (4) including Kearns–Sayre (5,6,7). In order to determine the specificity of the deletions in KSS we have studied the mitochondrial DNA organization of 21 additional cases and different tissues of the same case.

MATERIAL AND METHODS

Proteinase K, restriction enzymes and nick-translation kit were from Boehringer Mannheim (^{32}P) dCTP (3000 Ci/mmole) and Hybond C filters were from Amersham France. Agarose was from Bethesda Research Laboratories (BRL), chemicals were from Sigma and from Merck.


Xba I
Sac I
Pvu II
Pst I
Kpn I
Hpa I
Hind III
Eco RI
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13
14
15
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xl d
xl c
7440
H A
8286
H B
8729
9020
H C
9021
10254
xl Bc8

Patients.
The patients were selected according to criteria defining KSS with unaffected relatives (8). Biopsies of deltoid muscle were frozen until analysis. The brain, spinal cord and muscle specimen of a patient deceased in 1983 and described by Robain et al. (9) were stored frozen until analysis.
Tissues.
Trypsinized skin was grown in MEM supplemented with 10% (v/v) bovine serum. At the second passage, fibroblasts were trypsinized and their DNA extracted with proteinase K, phenol extraction and ethanol precipitation; the lymphocytes were purified on Ficoll−Hyplaque gradient.
Extraction of total DNA.
The DNA from 30−50 mg of tissue was extracted by overnight digestion with 100 μg/ml of proteinase K in 0.5 ml of 40 mM Tris-HCl , pH 8.0, 0.4 M NaCl, 10 mM EDTA, 0.5% SDS at 37°C. DNA was recovered by extraction with phenol, phenol/chloroform, precipitated with 2 volumes of ethanol and 0.1 M NaCl, and dissolved in 50 μl of Tris-HCl, pH 8.0, 1 mM EDTA.
Southern analysis.
0.5 μg samples of DNA were subjected to endonuclease restriction with a 10 fold excess enzyme units, then separated by electrophoresis (8V/cm−5 hours) on a 0.8% agarose gel, stained 15 min. with 0.1 μg/ml ethidium bromide and subjected to Southern blotting as previously described (10).
Mitochondrial DNA probes.
Human mtDNA from placenta was purified according to Drouin (11). The $XbaI_{3-4}$ fragment (probe A)(map position 7.440-8.286) encoding the C terminal part of subunit I of cytochrome C oxidase , $tRNA^{Ser}$, $tRNA^{Asp}$ and subunit II of cytochrome C oxidase was cloned into PUC 19 digested with XbaI. The MboI fragment (map position 8.729-10.254)(3) was cloned into the BamHI site of vector SP65. Further digestion of the recombinant plasmid by EcoRI and PstI yields fragment B of 292 bp containing part of the gene encoding ATPase 6 (map position 8.729-9.020) and fragment C of 1.234 bp (map position 9.020-10.254) containing part of the genes for subunit III of cytochrome C oxidase and subunit 3 of NADH dehydrogenase . MtDNA probes from Xenopus Laevis were kindly provided by Pr. J.C. Mounolou (12).

RESULTS

The total DNA from tissues samples were subjected to endonuclease restriction, then analyzed by Southern blot using mtDNA as a probe. The action of PvuII at coordinate 2.650 (3) or of BamHI (14.258) with the exception of some known morphes (13) normally produces a single mtDNA species of 16.569 bp if one homogeneous mtDNA population contains that restriction site (Fig.1). If two populations differ by their length and still contain the restriction site, they will be linearized and separated according to their migration rates. In some cases, the 2 mtDNA populations, differing in length and absence of single restriction site give the appearence of uncompletely linearized molecules (undigested) in addition to

Fig. 1 *Linearized representation of the human mitochondrial DNA restrictions map.* Numbers from 1 to 16 indicate 1.000 base pairs according to the sequence described by Anderson et al (1981). Each restriction enzyme is shown on the left with their cleavage position. The DNA probes are in the lower part of the diagram with their exact position. H for human, XL for Xenopus Laevis mt DNA probes A, B, C and BcB refer to their nomenclature on the Southern blot of figure 2.

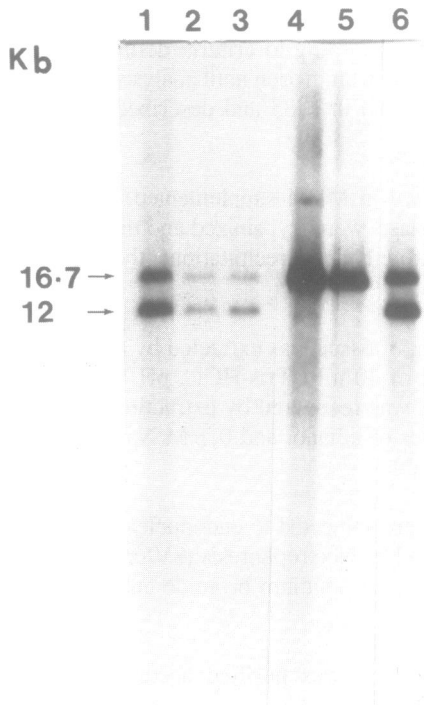

Fig. 2 *Deletion mapping with mtDNA probes by Southern analysis.* The BamHI digested DNA from the muscle of a KSS patient was separated transferred and then hybridized with specific mtDNA probes: lane 1: X.Laevis 15.348-480 (probe BcB); lane 2: X.Laevis 2.480-4.541 (probe D); lane 3: X.Laevis 5.801-8094 (probe C); lane 4: human 8.729-9.020 (probe B); lane 5: human 9.020-10.254 (probe C); lane 6: human 7.440-8.286 (probe A).

the normal sized one. This protocol was used for detecting large deletions and their approximate mapping.

Heteroplasmic mtDNA deletion mapping.

Mitochondrial DNA probes were used for hybridization with the total muscle DNA subjected to endonuclease restriction BamHI. The autoradiogram of figure 2 shows that the mtDNA species hybridize with the Xenopus Laevis mtDNA probes containing nucleotides 15.348 to 480 (lane 1), 2.480 to 4.541 (lane 2), 5.801 to 8.094 (lane 3), as well as human as mtDNA probe A containing nucleotides 7.440 to 8.286 (lane 6). On the other hand, while probe B containing the ATPase gene (8.729-9.020) or probe C containing genes for subunit III of cytochrome C oxidase and 3 of NADH dehydrogenase (9.020-10.254) hybridize with normal sized mtDNA, they showed no signal with the 12 Kb species (lanes 4 and 5 respectively) which thus lack the corresponding DNA sequences. The localization of the deletion was then investigated more precisely by comparing the restriction endonuclease pattern obtained from normal sample, with the DNA from the muscle of the patient as shown on figure 3. The 12 Kb additional mtDNA species from the muscle of a KSS patient also appears after restriction with HindIII, EcoRI and PstI, it corresponds to a linear mtDNA molecule that have lost other restriction sites at positions 11.680 and 12.570 for HindIII, position 12.640 for EcoRI and 9.020 for PstI.

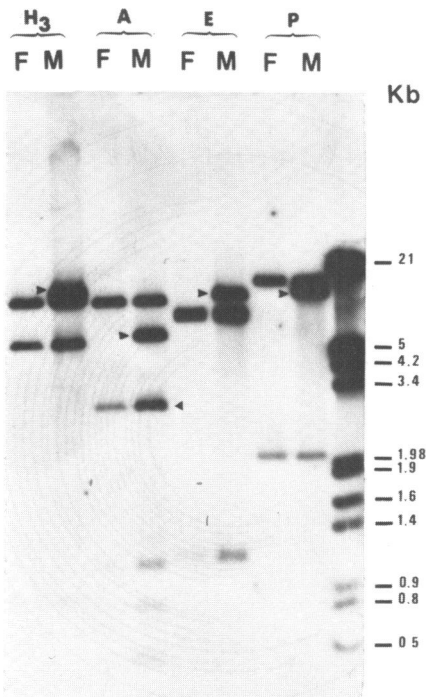

Fig. 3 *Deletion mapping of the mitochondrial DNA using various restriction endonucleases.* The figure shows a comparison of restriction site data between non heteroplasmic mtDNA (F), and heteroplasmic mtDNA with deletion from the muscle of a patient (M). The arrow indicates the additional DNA fragment resulting from the digestion of the deleted genome by HindIII (H3), AvaII (A), EcoRI (E) and PstI (P). The longer restriction fragment observed with HindIII corresponds to the linearized deleted molecule which has lost two of three restriction sites, the HindIII 2 and 3 sites. The AvaII intermediate fragment corresponds to the $AvaII_{5-7}$ minus the 4,7 Kb deletion (loss of $AvaII_6$ restriction site). —Additional $AvaII_{7-8}$ and $AvaII_{2-3}$ fragments present in the normal molecule are also shown. The EcoRI digestion shows the presence of $EcoRI_{1-2}$ fragment as well as the longer $EcoRI_{2-1}$ resulting from the loss of $EcoRI_3$, and the deletion located in that fragment. The PstI additional lower band corresponds to the $PstI_{2-1}$ fragment minus deletion.

Furthermore restriction with AvaII yields one additional 6 Kb molecule and $AvaII_{7-8}$ fragment (3.023 bp) compatible with a restriction site loss at position 12.629 and a 4,7 Kb deletion between sites $AvaII_5$ and $AvaII_{6-7}$. Additional restriction with XbaI (not shown) indicates the presence of $XbaI_4$ (position 8.286) restriction site but loss of $XbaI_5$ (position 10.256).

We can infer from these data that the left border of the deletion maps between nucleotides 8.286 and 9.020 ($PstI_2$ loss) and the right region is located between nucleotides 12.640 ($EcoRI_3$ loss) to 13.367 ($AvaII_7$ present). Though uncertainty concerns with the presence of the gene encoding $tRNA^{Lys}$ and subunit 8 of ATPase, the deleted genes encoded subunit 6 of ATPase, subunit III of cytochrome C oxidase, subunits 3, 4L, 4 and part of subunit 5 of NADH dehydrogenase complex (14), as well as $tRNA^{Gly}$, $tRNA^{Arg}$, $tRNA^{His}$, $tRNA^{Ser}$ and $tRNA^{Leu}$ genes.

Muscle biopsies from 21 other KSS patients were analyzed using the combination of previously described methods. In all studied cases, heteroplasmic mtDNA deletions were

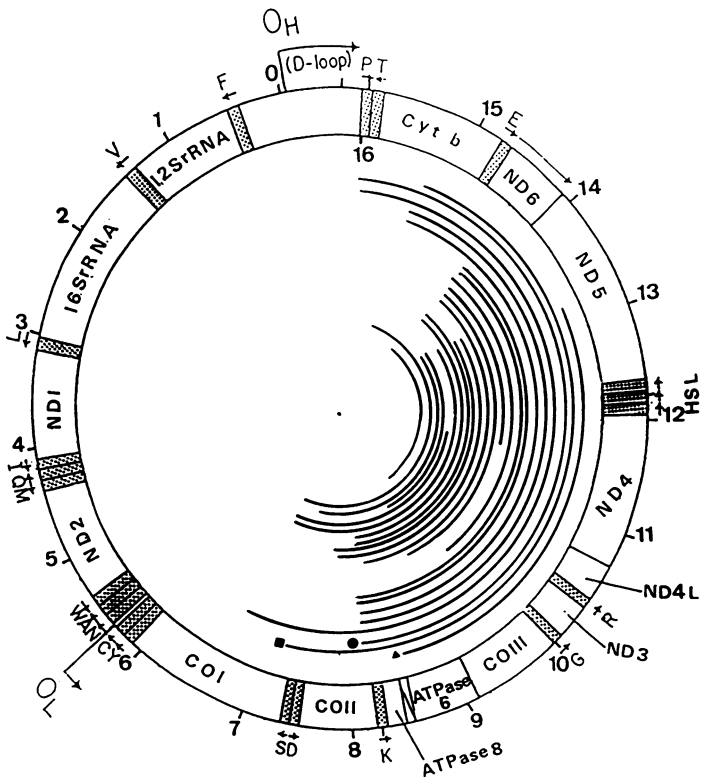

Fig.4 *Summary of the muscle mtDNA deletions mapping in 22 patients with Kearns–Sayre Syndrome.* The heavy and light strand origins of DNA replication are indicated by OH and OL. Dotted segments with letters show tRNA genes with their specificities. Genes for ND1 to ND6 represent subunits of the NADH dehydrogenase (complex I) (15, 20); ATPase : ATP synthetase subunit genes (complex V); Co: cytochrome C oxidase subunit genes (complex IV); rRNA: ribosomal RNA genes and cyt b: apocytochrome b gene. The borders of the deletions are shown with an accuracy of 0,4 Kb.

Symbols: triangle: Deletion found in the muscle and primary grown fibroblasts. Square: Deletion found in the muscle, the skin and the lymphocytes. Circle: Deletion found in the muscle, the spinal cord and the cortex.

found, some of them encompassing more than the half of the mitochondrial genome. The deleted region not only contains indispensable tRNA genes for mitochondrial mRNA translation but also open reading frames for the normal mitochondrial function.

Tissue specificity.

Previous results assigned heteroplasmic mtDNA deletions to the muscle tissue, which could be interpreted as being the result of its specific mode of organogenesis by fusion of myoblasts containing the normal mtDNA, with other myoblasts containing deleted mtDNA. Tissue specimens from the spinal cord and from the cortex of a deceased KSS patient were subjected to the same analysis as its muscle mtDNA. As shows figure 5, the mtDNA from these three tissues are indistinguishable, and display the same heteroplasmy. Also the skin, the primary grown fibroblasts (1 month) and the lymphocytes, from another patient with a 7,5 Kb deletion, display the same heteroplasmy as in the muscle. Thus deletions are unlikely

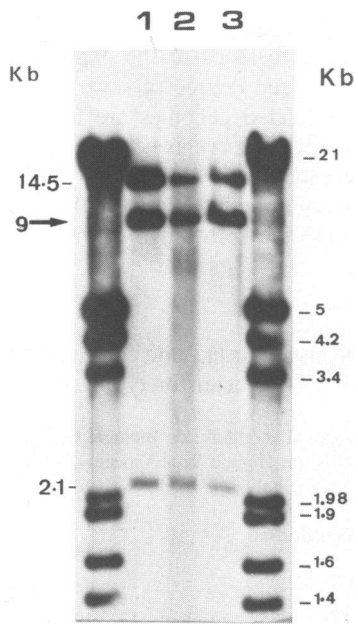

Fig. 5 *Southern blot analysis of the mitochondrial DNA, from different tissues of a KSS patient, digested by PstI.* Lane 1 : muscle, lane 2 : spinal cord, lane 3 : cortex.

to result from post embryogenesis events in these tissues but probably occur before the blastula stage when tissue cells undergo differentiation into mesoderm and ectoderm.

DISCUSSION

These data confirm our earlier finding of mitochondrial DNA deletion in patient presenting Kearns–Sayre Syndrome (5) which was confirmed by others (6, 7). Except in myopathies where deletions occur as well in the ribosomal genes (4), all the deletions found presently map in a potentially single stranded region during H strand replication (15). We thus hypothesize that deletion mechanisms involved in these patients result from the asymetric replication cycle of the mammalian mitochondrial genome. The presence of the normal sized molecule with the deleted constitutes heteroplasmy, an observation first reported on mice (16). Heteroplasmy with gene duplication in the non coding sequence of Drosophila (17) and structural genes in myopathies (18) have also been reported.

The presence of the normal sized molecule is a prerequisite for the maintainance of a threshold DNA function in mitochondria (19). Heteroplasmy with variable deletions between individuals allows a tRNA complementation at the translational level; on the other hand competition of each genome for replication and transcriptional factor may produce the various phenotype an onset of the disease in that genetic disorder.

The replication rate favours, at the kinetic level, the deleted genome which is indeed predominant in most of the studied tissue cases. On the other hand, a selection pressure operates on the deleted genome because of the lack of tRNA genes, unless intramitochondrial complementation by the normal genome, occurs. Such a translational defect in mitochondria, harboring the deleted genome, may lead to an energy supply defect that induces a reduction

of the importation of proteins involved in the replication of the deleted mtDNA. It may be therefore anticipated that an imbalance of the expression of each genome may be responsible for the variable onset and degree of severity of KSS.

It is also interesting that the deleted genomes are found in brain, muscle, skin and lymphocytes which suggests that deletion events occur before the gastrula stage when these tissues undergo separate differentiation.

Further studies of the expression of both genomes at the transcriptional and translational levels may give new insights into the knowledge of the development of Kearns–Sayre Syndrome symptoms.

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