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Mitochondrial genome expression in a mutant strain of *D.subobscura*, an animal model for large scale mtDNA deletion

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ABSTRACT

A mitochondrial mutant strain of *D.subobscura* has two mitochondrial genome populations (heteroplasmy) : the first (20 – 30% of the population, 15.9 kb) is the same as could be found in the wild type; the second (70 – 80% of the population, 11 kb) has lost by deletion several genes coding for complex I and III subunits, and four tRNAs. In human pathology, this kind of mutation has been correlated with severe diseases such as the Kearns-Sayre syndrome, but the mutant strain, does not seem to be affected by the mutation (1).Studies reported here show that: a) Transcripts from genes not concerned by the mutation are present at the same level in both strains. b)In contrast, transcript concentrations from genes involved in the deletion are significantly decreased (30 – 50%)in the mutant. c) Deleted DNA was expressed as shown by the detection of the fusion transcript. d) The mtDNA/nuc.DNA ratio is 1.5 times higher in the mutant strain than in the wild type. The mutation leads to change in the transcript level equilibrium. The apparent innocuousness of the mutation may suggest some post-transcriptional compensation mechanisms. This drosophila strain is an interesting model to study the consequence of this type of mitochondrial genome deletion.

INTRODUCTION

Certain types of myopathies and encephalomyopathies are correlated with mitochondria and mitochondrial genome alterations (2–4). These may be of several kinds : point mutations involving protein coding genes or tRNA (5–9), or more substantial deletion (10–12) or duplication type mutations (13), localized by most studies in a highly specific single-strand zone during replication (14–16). According to their localization, these deletions or duplications frequently provoke major respiratory chain function defects, with consequent cellular energy supply deficiencies. They may thus account for observable clinical manifestations.

Studies in this field present a number of inherent drawbacks: they deal with isolated manifestations and random clinical signs, in an area where experimental possibilities are limited (human mutation). An animal model should allow the development of a different approach to the problem.

A *Drosophila subobscura* strain discovered in the natural environment (1) possesses a heterogeneous population of mitochondrial genomes (heteroplasmy). One fraction of this population (20 to 30%) is of the wild type, and the remaining fraction (70 to 80%) contains a substantial deletion in the coding part. The mutation is similar to those encountered in human pathologies such as the Kearns–Sayre syndrome. Yet despite the handicap ensuing from the mutation, and contrary to observations in human pathology, the strain appears to be unaffected.

Our first studies of this mutant strain analyzed mitochondrial DNA transcription, and assessed relative concentrations of the various mitochondrial transcripts. These concentrations were compared with levels measured in mitochondria of the wild-type strain, bearing 100% intact mitochondrial genomes.

Our results showed concentrations to be identical for transcripts of mitochondrial genes unaffected by mutation, but lower for transcripts of affected mitochondrial genes in the mutant. Detection in the heteroplasmic strain of a new transcript whose size matched that expected of the fusion transcript indicated that the deleted genomes were indeed transcribed. The possible implications of these results on the mutant strain phenotype are discussed.

MATERIALS AND METHODS

Isolation of mitochondria

Mitochondria from adults flies (1 to 8 days) were isolated as previously described (17).Mitoplasts were obtained by incubation of mitochondria with digitonin 100μg/mg protein (17).

Total DNA preparation from whole flies

Whole flies (100 to 200 mg) were crushed in 1 ml of extraction buffer (Tris 10mM pH 7.8, NaCl 60 mM, EDTA 10mM) at 0–5°C to which was added 1 volume of lyse buffer (Tris 300 mM, pH 9, EDTA 100 mM, DEPC 0.8%), SDS 1%, Proteinase K 100μg/ml and incubated 1 h at 65°C. K acetate 8M (1/10 vol.) was then added and mixture was incubated for 45 min. at 0–5°C

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and centrifuged at 10Kg for 15 min. 1 volume of 2-propanol was added to the supernatant which was left at room temperature for 30 min. and centrifuged 10 Kg, for 10 min. The pellet was dissolved in 0,5 ml buffer Tris 10mM pH 7.4, EDTA 1mM (TE 1×), treated with RNAse A 200μg/ml 30 min. at 37°C, and extracted twice with phenol-chloroform. DNA was precipitated from the aqueous phase by 2.5 volume ethanol. DNA pellet was washed with ethanol 70% and dissolved with 200 to 500μl TE 1×.

RNA extraction from mitoplasts

Mitoplasts were incubated 30 min at 37°C. with the lysis buffer (NaCl 0.1M, Tris 10mM pH 7.4, EDTA 1 mM, Proteinase K 100 μg/ml, Sodium dodecyl sulfate 1%, RNAsine (Boehringer) 100 u/ml).The mixture was extracted twice with phenol-chloroform and precipitated with ethanol. The pellet was dissolved in 100 μl DEPC 0.8% water and treated with 200 U HPLC grade DNAse 1 (Pharmacia) in buffer Tris 50 mM (pH 8), $MgCl_2$ 10mM, NaCl 50 mM with 200 U/ml RNAsine at 37°C, 30 min, then extracted with phenol-chloroform. After ethanol precipitation, the RNA pellet was dissolved with TE 1×.

Electrophoresis of nucleic acids and hybridization

RNA and DNA electrophoresis, blotting and hybridization were done as described previously (18). In some case (slot-blot), DNA was applied directly to nylon membrane (Gene-screen DUPONT) with a slot apparatus and then hybridised as described for Southern blot.

Quantification of hybridization

Northern or Southern blot hybridization signals were analysed by densitometry with an Ultroscan laser system (LKB).

In order to compare signals hybridization in Northern experiments, srRNA was used as an internal control. The same membrane was used for all hybridizations and the ratio (studied $transcript_H$/studied $transcript_{W)}$ versus ($srRNA_H$ /srRNA W) were calculated.

In slot-blot experiments with total DNA, the ratios (signal obtained with mitochondrial probe/signal obtained with nuclear probe) were calculated for the two strains and compared.

Probes used

All probes were obtained from *D.melanogaster* DNA. They were cloned in pGEM7 Zf or pGEM3 Zf. Nucleotide positions are referred as described by CLARY and WOLSTHENHOLME (19) for the probes srRNA, HE 143 and CO III and by GARESSE (20) for the probes SE 162, Cyto b and ND4.

srRNA : A 349 bp fragment obtained by Cla I and Hind III digestion (14490−14141). This clone revealed the srRNA transcripts (750 n).

HE 143 : A 1450bp fragment obtained by Hind III and Eco R1 digestion (5696−7146). Transcription of this clone with T7 polymerase in vitro yields an antisens riboprobe of ND5 transcripts (1800n.).

COIII : A 711 bp fragment obtained by AccI and PvuII digestion (5454−4743). This clone revealed CO III transcripts (825n.)

SE 162 : A 1617 bp fragment obtained by Sna B1 and Eco R1 1 digestion (7244−5627). It revealed transcripts from ND1 (1000n.) and Cyto b (1200n.)

Cyto b : A 851 bp fragment obtained by Pvu II and Sst I digestion (10674−11525) .This probe revealed Cyto b transcripts.

ND4 : A 915 bp fragment obtained by XmnI digestion (8430−9345). This clone revealed the ND4-ND4L transcripts (1700n.).

Probes are labelled with ^{32}P dATP by nick translation or with ^{32}P UTP by *in vitro* transcription with T7 polymerase (HE 143).

RESULTS

Measurement of the heteroplasmy

As described by A.Volz et al. (1), the deleted zone (Figure 1) extended from nucleotide 6943 (in the ND1 gene) to nucleotide 11874 (in the ND5 gene), with reference to the *D.yakuba* mtDNA sequence (19). Five coding genes for complex I subunits were thus either truncated (ND1, ND5) or lost completely (ND4, ND4L, ND6). The coding gene for the single complex III subunit of mitochondrial origin, cytochrome b, was also implicated in the deletion, together with four tRNAs (Ser, Threo, His, Pro).

The extent of heteroplasmy was evaluated by Southern blotting on total DNA obtained from adult flies (1−7 days) after cutting with the Msp1 enzyme and hybridization with the CO III probe (Figure 2). The probe recognized two specific fragments as either intact mitochondrial genomes (4.3 kb) or deleted genomes (3.3 kb). Densitometric measurements indicated 70 to 80% heteroplasmy corresponding to deleted DNA.

Other measurement methods (not shown), such as analyzing electrophoresis gels obtained by staining mitochondrial DNA of both strains with ethidium bromide after cleavage with a restriction enzyme (*Hind* III), or radioactive labelling by filling in the end of the molecule, showed 70−80% of the deleted mtDNA population in the mutant strain.

STUDY OF MITOCHONDRIAL TRANSCRIPTION

The equilibrium transcript level was estimated after RNA extraction from isolated adult flies mitochondria, or from total RNAs. The transcripts were analyzed by Northern blotting with various probes.

Transcripts of genes out the deletion. Two probes situated on each side of the deletion zone were used: the srRNA probe and the CO III probe (Figure 1). Northern blot results are showed in Figure 3.

Same amounts of mtRNAs (in μg) derived from heteroplasmic (H) or wild-type (W) strains were used. RNAs extracted from the *D.melanogaster* strain (M) was also used as control. The srRNA and CO III transcripts of this strain have already been identified (18).

Signals obtained after hybridization of the RNA extracted from the two strains (RNAs H/RNAs W ratios) were compared as described in Material and Methods. Results of densitometric analysis performed for different extractions (four experiments) indicated that these ratios were identical for CO III and srRNA.

To confirm these results, whole RNA fractions extracted from both strains and treated with 100 μg of DNAse 1 (to eliminate DNA contamination) were directly hybridized (slot-blot) with srRNA or CO III mitochondrial probes and with the nuclear probe DNA 45S (21) (not shown). The srRNA/45S RNA hydridization signal ratios were identical for both strains.

These results confirm that transcripts of genes not implicated in deletion attained identical steady-state levels in the two strains.

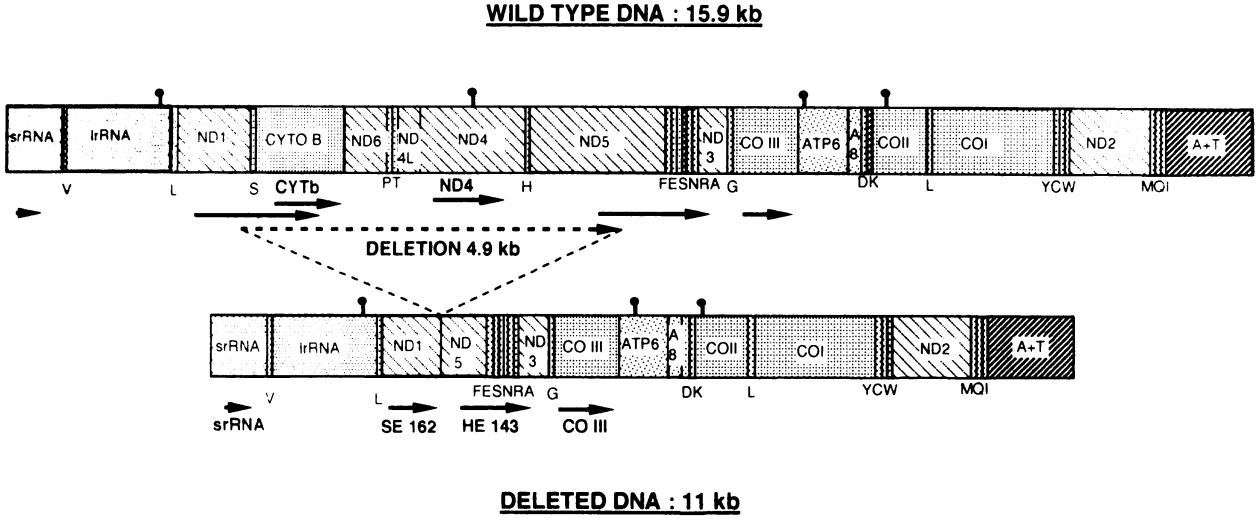

Figure 1. Wild type and deleted mitochondrial genome maps. Arrows indicate the location of the different probes used.(↑) MspI sites. The deletion leads to the fusion of the 5′ region of ND1 and the 3′ region of ND5. A+T: A+T rich region.

Transcripts of the genes implicated in mutation. Transcript concentrations for genes in that deleted DNA were assessed by hybridization with various probes (Figure 1), which either overlapped the deleted zone (SE 162, HE 143) or were completely included thereby (ND4, cytochrome b).

Transfer filters of mtRNAs extracted from both strains were hybridized with these probes, but also with the srRNA probe (internal control). Results are presented in Figures 4 and 5. Relative percentages of each transcript were estimated with reference to srRNA. Transcript/srRNA hybridization signal ratios were compared for the wild-type and mutant strains. The same filter underwent successive hybridization with the SE162 and srRNA probes (Figure 4A), prior to dehybridization then rehybridization with the cytochrome b probe alone (Figure 4B).

Figure 4A shows four visible bands in the lane containing isolated H strain transcripts : 750n. corresponding to srRNA (18), 950 n. (ND1), 1200 n. (Cyto b) and 1350 n. Hybridization with the cytochrome b probe alone (Figure 4B) confirmed that the 1200 n. band corresponds to the transcript of this gene.

Only three bands were detected in the lane containing isolated W strain transcripts (srRNA, ND1, Cyto b), the 1350 n. was not detected.

The same filter was used for hybridization with the riboprobe transcribed from HE 143 probe by T7 polymerase (Figure 5A) or for hybridization with the ND4 probe (Figure 5B).

Figure 5A shows two bands for RNA isolated from the H strain: one at 1800 n. corresponding to ND5 transcription, and one at 1350 n. Only the 1800 n. band was visible in the W transcript lane. In Figure 5B, only one band was detected in each of the two lanes : 1700 n., corresponding to the single transcript of the ND4 and ND4L genes.

Five experiments identical to those described above were performed. R_H/R_W ratios were calculated as described in Material and methods. The lowest ratio was 0.35(+/−0.13)for ND5. These ratios were 0.45(+/−0.15)for ND1 and 0.55(+/−0.11) for ND4-ND4L). The highest ratio (0.66 (+/−0.11)) was obtained for the cytochrome b transcript.

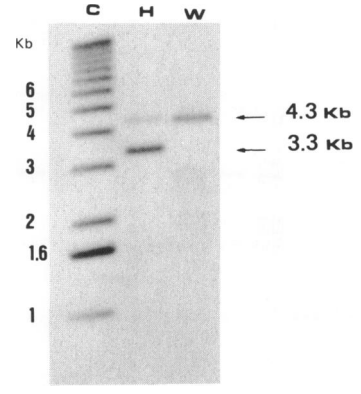

Figure 2. Southern blot analysis of the mitochondrial genome. 15 μg of DNA fraction extracted from wild type (W) or mutant (H) adult flies were digested by MspI, electrophorezed on 1% agarose gel, blotted on membrane and hybridized with COIII probes. C: size marker.

Fusion transcript. Autoradiographies of hybridized H strain transcripts with probes overlapping the deleted zone (SE162 and HE143) showed a new band at 1350 n. (Figures 4A and 5A). This was not observable with transcripts isolated from the wild-type strain, nor with the cytochrome b or ND4 probes hybridized with transcripts from the mutant strain (Figures 4B and 5B).The size of this band corresponds to the predicted size of the transcript resulting from ND1-ND5 gene fusion, i.e. 1328 nucleotides (1). Measurement of the fusion transcript hybridization signal showed it to be identical to that of the ND1 transcript.

Estimation of relative mitochondrial genome levels in the mutant strain

The quantity of mitochondrial DNA isolated from the H and W strains was estimated with reference to nuclear DNA. The extracted nucleic acids from whole flies were treated with RNAse A for RNA elimination, then directly deposited on a membrane (slot-blot) before hybridization with either a nuclear probe (DNA

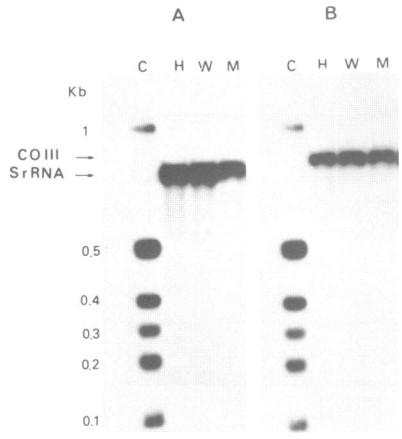

Figure 3. Northern blot analysis of mtRNA hybridized with probes srRNA and CO III. RNA fraction extracted from wild type (W), mutant strain (H) or from *D.melanogaster* (M) were fractionated on 1,3% agarose gel, blotted, and hybridized with srRNA probe (A), or COIII probe (B). C: size marker.

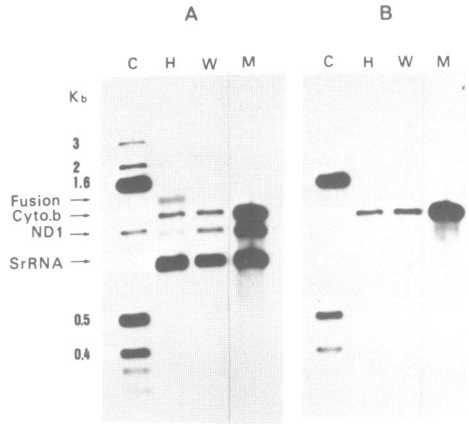

Figure 4. Northern blot analysis of mtRNA hybridized with probes SE 162 and srRNA or CytoB. RNA fraction extracted from wild type (W), mutant strain (H) or *D.melanogaster* (D) were fractionated on agarose gel, blotted, and hybridised with probes SE 162 and srRNA (A). The membrane was then dehybridized and rehybridized with the probe CytoB (B). C: size marker.

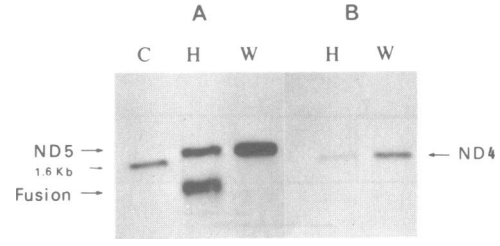

Figure 5. Northern blot analysis of mtRNA hybridized with probes HE143 (A) or ND4 (B). The same membrane described in Figure 4 is hybridized with (A) riboprobe of HE 143 probe (ND5 antisens) or with ND4 probe. W: wild type RNA, H: mutant strain RNA. C: size marker.

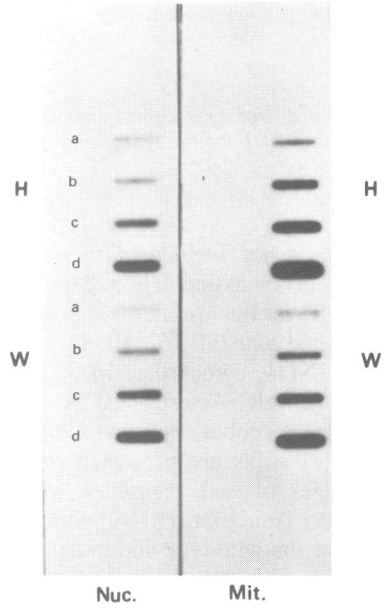

Figure 6. Slot blot analysis of DNA extracted from both strains and hybridized with nuclear and mitochondrial probes. Increasing amounts (a:0,25, b:0,5 c:1, d: 2 μg) of DNA extracted from wild type (W) or mutant strain (H) were loaded on a membrane and hybridized with the nuclear probe rDNA 45S (Nuc.), or with the mitochondrial probe, srRNA, (Mit). Hybridization signals were analyzed by densitometry.

45S) or an mtDNA probe (srRNA). Results are shown in Figure 6. Ratios for the srRNA/DNA 45S hybridization signals obtained with the H and W strains were then compared. This ratio was 1.5 (+/−0.31) times higher in the mutant than in the wild type strain. Other nuclear probes (histone, β tubulin and actin probes) yielded identical results (not shown). Hence, there was an increase in the number of mitochondrial genomes per nuclear genome in the mutant strain.

DISCUSSION

According to the various measurements performed, 70 to 80% of the mitochondrial genomes showed deletion involving more than 30% of their coding fraction. Apart from the four tRNAs, which constitute unique entities on the mitochondrial genome, deletion bore on two respiratory complexes : complex III with cytochrome b, and complex I. This latter was particularly affected, 5 subunits being either totally absent (ND6, ND4 and ND4L) or severely truncated (ND1 and ND5). Cytochrome b plays a key role in electron transfer from ubiquinone to cytochrome c (22). Although their roles are less well known, complex I subunits also appear to be involved in the functioning of this complex. For example, ND1 is implicated in the rotenone bond and ubiquinone interactions (23), and ND4 may play a very important role in exchanges of reducing power with the mitochondrial matrix (25). The deletion also involved four essential tRNAs, possibly blocking all mitochondrial translation. Extensive, massive deletion of this kind would necessarily cause major perturbations to cellular function and energetic equilibrium.

Such mitochondrial genome alterations have been correlated with extremely severe human pathologies such as the Kearns−Sayre or Pearson syndromes (10−13).

One of the most remarkable observations concerning the mutant strain was the fact that it appeared very little affected by this

mutation. The strain was encountered in the natural environment (1), and its fertility rate (number of eggs) and number of offspring (larvae or imago) were apparently unaffected. Moreover, the selective value of the mutation was showed to be non-significant (1).

One of the hypothesis which could be proposed, attributes the lack of modification in mitochondrial genome expression to over expression of the intact genome population, or to a substantial increase in the overall concentration of mitochondrial genomes per nuclear genome (therefore in the intact mitochondrial genome concentration). These two possibilities may allow deletion-prone gene transcript concentrations to be kept at equivalent levels in both strains. Other hypotheses can also be postulated, such as over translation of transcripts of the involved genes.

The first step in genome expression, i.e. transcription, was studied in order to test these hypotheses. Results showed that:
a) For genes not involved in deletion, equilibrium transcript concentrations were identical in the mutant and wild-type strains.
b) Conversely, equilibrium transcript concentrations were affected for genes involved in deletion : there was a 45 to 65% decrease for genes coding for NADH-dehydrogenase subunits, and a 35% decrease for the cytochrome b gene.
c) The presence of a new 1350 n. transcript, identified in the mutant strain by probes in borderline deletion areas, indicated transcription of the deleted genomes.
d) Relative concentrations of mitochondrial genomes with reference to the nuclear genome increased about 50% in the mutant strain.

Hence, deletion clearly led to a modification in equilibrium transcript levels in the heteroplasmic strain for genes involved in deletion. There was no compensation by intact genomes, but the decreases in concentration were less than expected based on heteroplasmic levels. Indeed, had these two phenomena been directly proportional, the decreases should have amounted to 80% for the transcripts of all involved genes. These differences may be due to the relative increase in mitochondrial genomes in the mutant strain (mtDNA/nuclear DNA$\times$1.5 compared with the wild-type strain). Since the number of intact genomes per nuclear genome increased, the relative concentration of genes involved in deletion (per nuclear genome, thus per cell) likewise increased. As this increase amounted to 50%, the relative concentration of involved genes rose from 20 to 30%. The theoretical decrease in the concentration of involved gene transcripts (70%) was nevertheless lower than the measured concentration : 45$-$55% for ND1 and ND4-ND4L transcripts, and 35% for the cytochrome b gene transcript. There was thus no direct relation (perhaps except for ND5) between the quantity of mitochondrial genes and the equilibrium concentration of their transcripts. This implies partial transcriptional or post-transcriptional compensation for these genes.

The presence of the fusion transcript of the ND1 and ND5 genes indicated that deleted genomes indeed underwent transcription. Therefore, the transcripts of genes not involved in the deletion are presumably derived from both genomes. If mtDNA per nuclear genome was increased by 50% in the mutant strain, the concentration of these transcripts should have increased by 50% as well. In fact they occurred in equal concentrations in the two strains. This implies a regulatory mechanism which maintains transcript concentrations of these genes at a constant level (constant number of transcripts per cell or per mitochondria ?). This mechanism apparently exerts a lesser action on transcripts of the deleted genes possibly because they are present in lower concentrations. Such a mechanism may directly control the actual transcription process, (initiation of transcription in the drosophila, as suggested by Berthier et al (18), may occurred by a mechanism different from that described in mammals (25)), or ensure post-transcriptional regulation of the concentration of the various transcripts (turnover) (26). Control of this kind may account for the observed differences between transcript concentrations of complex I and complex III genes. The relative concentration of the fusion transcript was low (comparable to that of ND1). Once more, this finding does not correlate with the relative deleted genome concentration. Either these genomes have a low level of expression, in contrast with observations in human muscle fibbers or cell studies (27$-$29), or this transcript is characterized by a very short half-life. Sequencing of the deletion breakpoint (1) indicates that a frame shift occurs between ND1 and ND5, and that this generates a stop codon on the first nucleotides of the conserved ND5 fragment. The stability of such a transcript may be jeopardized, hence its faster subsequent degradation.

Such partially compensatory mechanisms (increase in the mitochondrial genome amount, and possible modulation of transcript half-life) perhaps account for the discrepancy between heteroplasmy levels and equilibrium transcript concentrations. These concentrations always present a clear decrease however and seem to contradict the apparent lack of a mutant phenotype. Other post-transcriptional or post-translational compensatory mechanisms may be involved. Mitochondrial biochemistry and bioenergetics studies in the mutant strain should elucidate this question. Preliminary results (manuscript underway) indicate that activity of the implicated respiratory complexes may also be affected. The compensatory mechanisms, allowing the mutant strain to withstand deletion and restore balanced cellular energetic may implicated other metabolic pathways and other compartments than the mitochondrial alone. We are currently investigating different ways.

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REFERENCES

1. Volz-Lingenhohl A., Solignac M. and Spierlich D.A. (1992), Proc.Natl. Acad. Sci. USA. in press.
2. Wallace D.C. (1987). In: Medical and Experimental Mammalian Genetics: A perspective, edited by V.A. Mc Kusick, T.H. Roderick, J. Mori and N.W. Paul (Birth defects). **23**, 137$-$190. A.R. Liss, New York.
3. Wallace D.C. (1989). Trends In Genetics, **5**, 9$-$13.
4. Di Mauro S., Bonilla E., Zeviani M., Nakagawa M. and DeVivo DC. (1985). Annals of Neurology, **17**, 521$-$538.
5. Sato T. (1991) Progress in Neuropathology. **7**, 1$-$8.
6. Shoffner J.M., Lott M.T., Lezza A.M.S., Seibel P., Ballinger S.W. and Wallace D.C. (1990), Cell, **61**,931$-$937.
7. Wallace D.C., Singh G., Lott M.T., Hodge.A., Shurrt.G., Lezza A.M.S., Eelsal.S. and Nikoskelainen E.K. (1988), Science, **242**,1427$-$1430.
8. Howell N. and Mc Cullough D. (1990), Am. J. Hum. Genet., **47**, 629$-$634.
9. Goto Y.I., Nonaka I. and Horai S.(1990), Nature, **348**, 651$-$653.
10. HoltI..J., Harding A.E. and Morgan-Hughes J.A. (1988), Nature, **331**, 717$-$719.
11. Holt I..J., Harding A.E., CooperJ.M., Shapira A.V.H., Toscano H., Clark J.B. and Morgan-Hughes J.A. (1989), Ann. Neurol., **26**, 699$-$708.
12. Zeviani M., Moraes C.T., Di Mauro S., Nakase H., Bonilla E., Schon E.A. and Rowland L.P.(1988), Neurology, **38**, 1339$-$1346.

13. Rotig A., Cormier V., Blanche S., Bonnefont J.P.., Lederst F., Romero N., Schmitz J, Rustin P., Fischer A., Saudubray J. M. and Munnich A. (1990), J. Clin. Invest. **86**, 1601−1608
14. Shoffner J.M., Lott M.T., Voljavec A.S., Soueidan S.A., Costigan D.A. and Wallace D.C. (1989), Proc. Natl. Acad. Sci. USA, **86**, 7952−7956.
15. Lestienne P. and Ponsot G. (1988), Lancet, **1**, 885.
16. Degoul F., Nelson I., Amselem S., Romero N., Obermaier-Kusser B., Ponsot G., Marsac C., and Lestienne P. (1991) Nuc. Acid Res., **19**, 493−496.
17. Alziari S., Berthier F., Touraille S., Stepien G. and Durand R., (1985), Biochimie, **67**, 1023−1034.
18. Berthier F, Renaud M., Alziari S., and Durand R..(1986), Nucl. Acid. Res., **14**, 4519−4533.
19. Clary D.O. and Wolstenholme D.R. (1985), J. Mol. Evol. **22**, 252−271.
20. Garesse R. (1988), Genetics, **118**, 649−663.
21. Kidd S.J. and Glower D.M., (1980), Cell, **19**, 103−119.
22. Weiss H. (1987), Current Topics in Bioenergetics, **15**, 67−90.
23. Ragan I.C. (1987), Current Topics in Bioenergetics, **15**, 1−36.
24. Majander A., Huoponen K., Savontous M., Nikoskelainen E. and Wikstrom M. (1991), Febs Letters, **292**, 1−2.
25. Clayton D., (1991), Progress in Neuropathology, **7**, 47−55.
26. Chomyn A. and Attardi G. (1987), in 'Cytochrome systems ': Molecular Biology and Bioenergetics, edited by Papa S., Chance B. and Ernster L., 145−151. Plenum Press, N.Y.
27. Mita S., Schmidt B., Schon E.A., Di Mauro S. and BonillaE. (1989), Proc. Natl. Acad. Sci. USA, **86**,9509−9513.
28. Nakase H., Moraes C.T., Rizzuto R., Lombes A., Di Mauro. and Schon E.A. (1990), Am. J. Hum. Genet., **46**, 418−427.
29. Hayashi J.I., Ohta S., Kiruchi A., Takemitsu M., Goto Y.U. and Nonaka L. (1991), Proc. Natl. Acad. Sci. USA, **88**:,10614−10618.