Virchows Archiv A Pathol Anat (1993) 422:7–15

Virchows Archiv A
Pathological Anatomy and Histopathology

Different in situ hybridization patterns of mitochondrial DNA in cytochrome c oxidase-deficient extraocular muscle fibres in the elderly

J. Müller-Höcker[1], P. Seibel[2], K. Schneiderbanger[1], and B. Kadenbach[2]

[1] Institute of Pathology, Munich University, Munich, Germany
[2] Institute of Biochemistry, Marburg University, Marburg, Germany

Received July 13, 1992 / Received after revision September 10, 1992 / Accepted September 14, 1992

Summary. Previous studies have revealed an increase of cytochrome c oxidase-deficient fibres/cells in the skeletal and heart muscle of humans during ageing. The enzyme defect is due to a lack of both mitochondrial and nuclear coded enzyme subunits. In the present investigation in situ hybridization of mitochondrial DNA (mtDNA) has been performed on extraocular muscles of humans over 70 years of age to show whether mutated mtDNA with the so called common deletion of 4,977 basepairs at position 8,482–13,460 of mtDNA accumulates in the cytochrome c oxidase-deficient fibres. The cytochrome c oxidase-deficient fibres revealed different hybridization patterns: a normal hybridization signal with three different mtDNA probes, a reduced or lacking signal with all three probes indicating depletion of mtDNA and a selective hybridization defect with the probe recognizing the "common deletion" region of mtDNA as evidence of mtDNA deletion. The results suggest that during ageing defects of cytochrome c oxidase are associated with different molecular alterations of mtDNA. Deletion and depletion of mtDNA are not the only nor probably the leading mechanisms responsible for the loss of respiratory chain capacity during ageing. The normal hybridization signal in most of the cytochrome c oxidase-deficient fibres and the loss of mitochondrial and nuclear protein subunits indicate the involvement of other, especially nuclear factors.

Key words: Mitochondrial DNA – Ageing – Cytochrome c oxidase deficiency – Extraocular muscles

Introduction

Cytochrome c oxidase is the terminal enzyme of the respiratory chain catalysing the reduction of molecular oxygen to water in the mitochondria. The mammalian enzyme is composed of 13 subunits. The three larger subunits I–III are essential for the catalytic function. They are coded on mitochondrial DNA (mtDNA) and synthesized in the mitochondria. The ten smaller subunits IV–VIII are derived from nuclear DNA and most probably exert regulatory functions (Kadenbach et al. 1987). Deficiency of cytochrome c oxidase is a typical feature of mitochondrial encephalomyopathies (DiMauro et al. 1987), but occurs, although to a lesser degree, also during ageing in various tissues (Hansford 1983; Müller-Höcker 1989, 1990; Trounce et al. 1989; Byrne et al. 1991) including the extra-ocular muscles (Müller-Höcker et al. 1992).

MtDNA is a double-stranded, circular molecule of about 16.6 kb in size (Anderson et al. 1981) and contains very few non-coding regions in the D-loop segment. The genome encodes for 22 transfer RNAs (tRNA), 2 ribosomal RNAs and 13 proteins all being part of the respiratory chain enzyme complexes (Anderson et al. 1981; Attardi 1981; Chomyn et al. 1985; Attardi and Schatz 1988): 7 subunits of NADH-dehydrogenase (complex I), one subunit of ubiquinone cytochrome c oxidoreductase (complex III), three subunits (I–III) of cytochrome c oxidase (complex IV) and two subunits of ATP synthase (complex V). In mitochondrial encephalomyopathies a variety of mtDNA defects have been reported in the last 3–4 years: single or multiple small and large scale deletions as well as point mutations especially in tRNA genes, duplications and depletions (Holt et al. 1988; Lestienne and Ponsot 1988; Zeviani et al. 1988, 1989; Wallace 1989; Schon et al. 1989; Morgan-Hughes et al. 1990; Shoffner et al. 1990; Yoneda et al. 1990; Kobayashi et al. 1990; Goto et al. 1990; Otsuka et al. 1990; Cormier et al. 1991; Harding 1991; Moraes et al. 1991; Obermaier-Kusser et al. 1991; Tanaka et al. 1991; Yuzaki et al. 1989; Enter et al. 1991; Tritschler et al. 1992).

In chronic progressive external ophthalmoplegia and Kearns-Sayre syndrome – two closely linked variants of mitochondrial encephalomyopathies (DiMauro et al. 1985; Petty et al. 1986; Lombes et al. 1989) – the 4,977

Correspondence to: J. Müller-Höcker, Pathologisches Institut der Universität München, Thalkirchner Strasse 36, W-8000 München 2, Germany

basepair deletion ("common deletion") at nucleotide position 8,482–13,460 of mtDNA has been described in various tissues (Holt et al. 1989; Mita et al. 1989; Moraes et al. 1989; Nelson et al. 1989; Gerbitz et al. 1990; Larsson et al. 1990; Obermaier-Kusser et al. 1990; Shanske et al. 1990; Degoul et al. 1991). In both disorders typically focal-segmental defects of cytochrome c oxidase are present (Johnson et al. 1983; Müller-Höcker et al. 1983, 1985, 1986; Byrne et al. 1985; Yamamoto and Nonaka 1988; Romero et al. 1989). The common deletion but smaller and larger deletions also occur although at a lower frequency, as a physiological event during ageing, especially in post-mitotic cells such skeletal muscle, heart muscle and brain (Cortopassi and Arn-

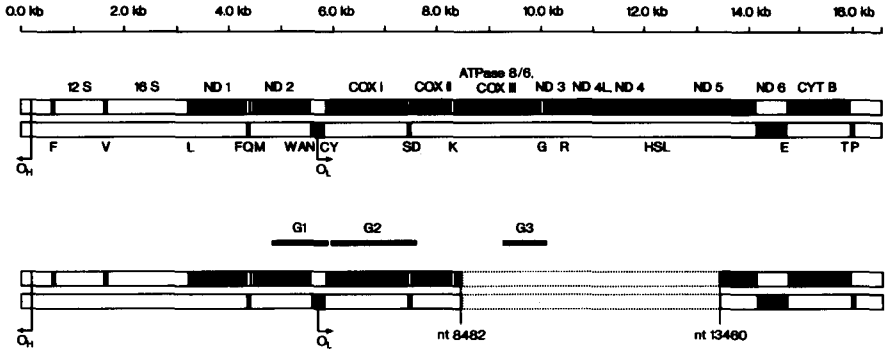


Fig. 1. Linearized map of mitochondrial DNA (mtDNA) with localization of mtDNa probes G1, G2, G3 employed in the study and of the 4,977 basepair deletion ("common deletion") at nucleotide position 8,482 on the left side and 13,460 at the right side of the mt genome. The following genes are indicated by boxes: ND 1–6 and ND 4 L of NADH dehydrogenase (complex 1), cytochrome b (*CYT B*, complex 3), COX I–III (subunits 1–3, complex 4), ATP 6, ATP 8 of ATP synthase (complex 5) large (16 S) and small (12 S) ribosomal RNA. O_H, O_L indicate origin of replication of the heavy and light strand. The transfer RNA genes are not shown

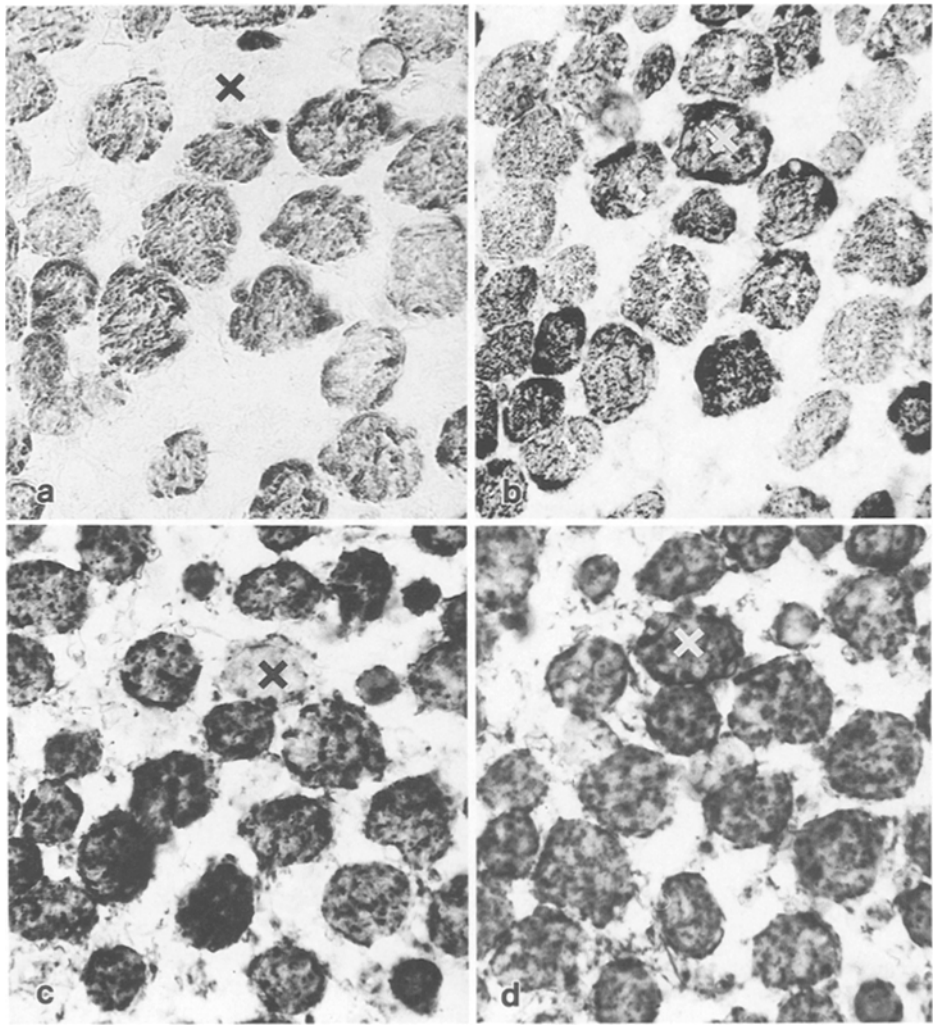


Fig. 2. Superior rectus muscle of a 72-year-old man. **A** Cytochrome c oxidase staining revealing a deficient fibre (*X*). **B** High succinate dehydrogenase activity in the deficient fibre (*X*). **C** In situ hybridization with the common deletion probe (G3) showing a selective defect in the enzyme-deficient fibre (*X*). There is some residual hybridization signal in the defective fibre, indicating co-existence of mutated and normal mtDNA. **D** In situ hybridization with a probe outside the deletion region (G1) exhibiting no defect (*X*). **A–D** ×640

heim 1990; Ikebe et al. 1990; Linnane et al. 1990; Hattori et al. 1991; Hayakawa et al. 1991; Katayama et al. 1991; Sugiyama et al. 1991; Zhang et al. 1992) but also in the liver (Yen et al. 1991) and other tissues (Linnane et al. 1990).

In situ hybridization and polymerase chain reaction (PCR) amplification have revealed that in mitochondrial disease the deleted mtDNA molecules accumulate in the cytochrome c oxidase-deficient fibres (Mita et al. 1989; Shoubridge et al. 1990; Collins et al. 1991), probably causing the enzyme defect when a certain threshold level of mutated mtDNA is reached.

In the present study, in situ hybridization of mtDNA was undertaken to clarify whether mtDNA molecules with the common deletion cluster in cytochrome c oxidase-deficient extra-ocular muscles of elderly subjects.

Materials and methods

Ten extra-ocular muscles (superior rectus) of humans over 70 years of age dying suddenly from cardiovascular disease, by accident or suicide were investigated not later than 20 h post-mortem by enzyme histochemical and immunocytochemical detection of cytochrome c oxidase as previously described (Müller-Höcker et al. 1989) and of succinate dehydrogenase (Lojda et al. 1976). In situ hybridization of mtDNA was also performed on frozen sections. Immunohistochemistry was performed with subunit specific antisera raised in rabbits against the mitochondrially derived subunits

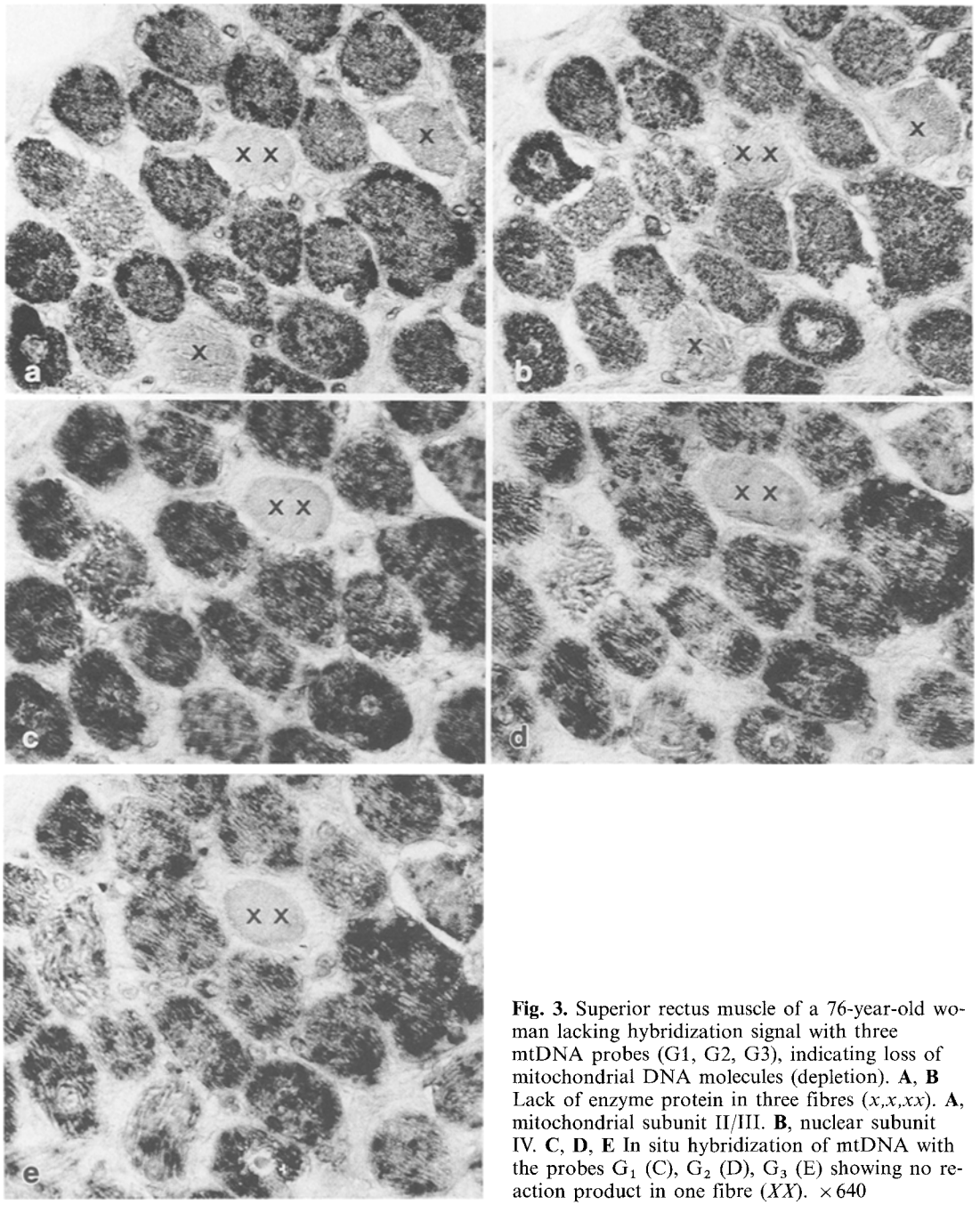
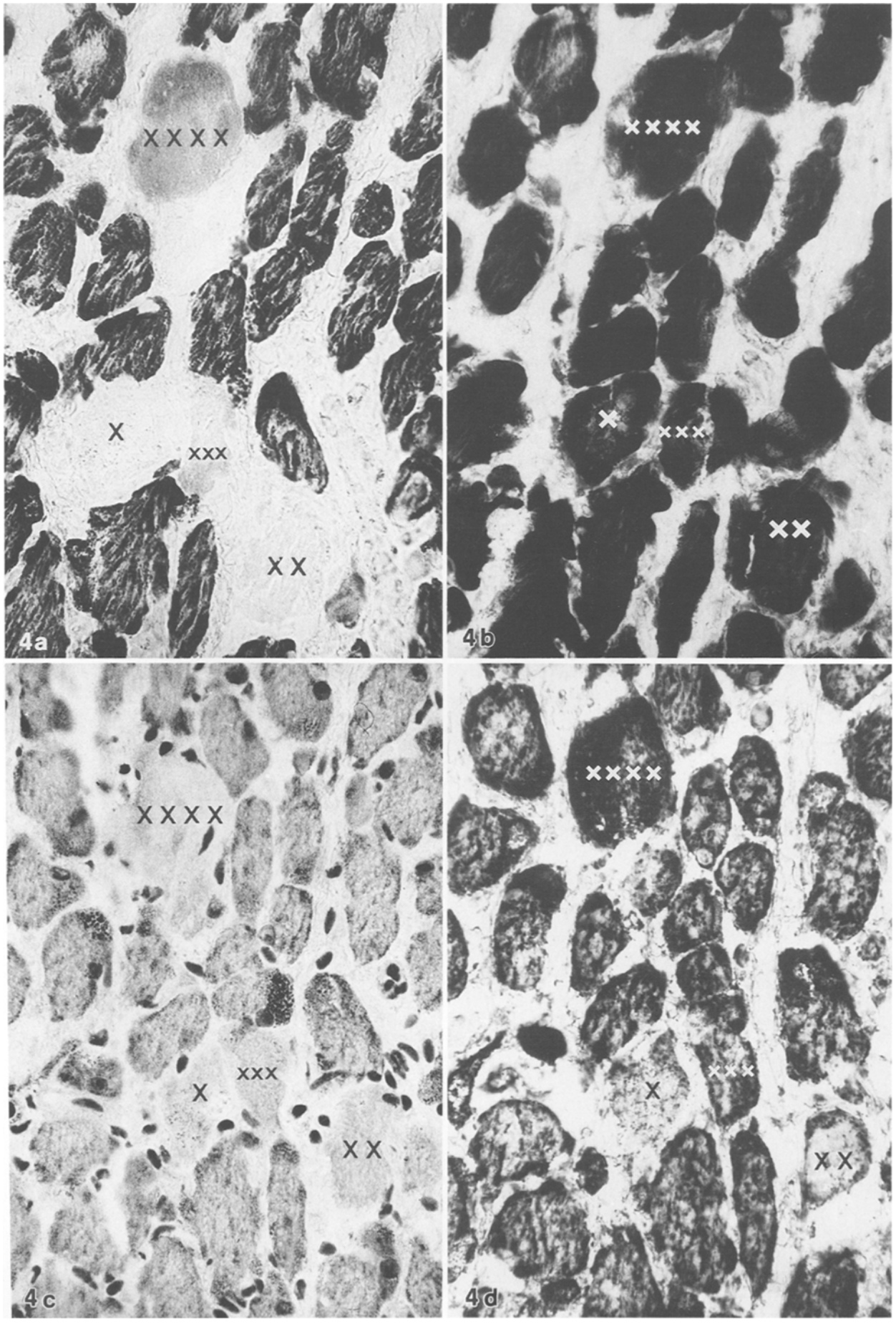
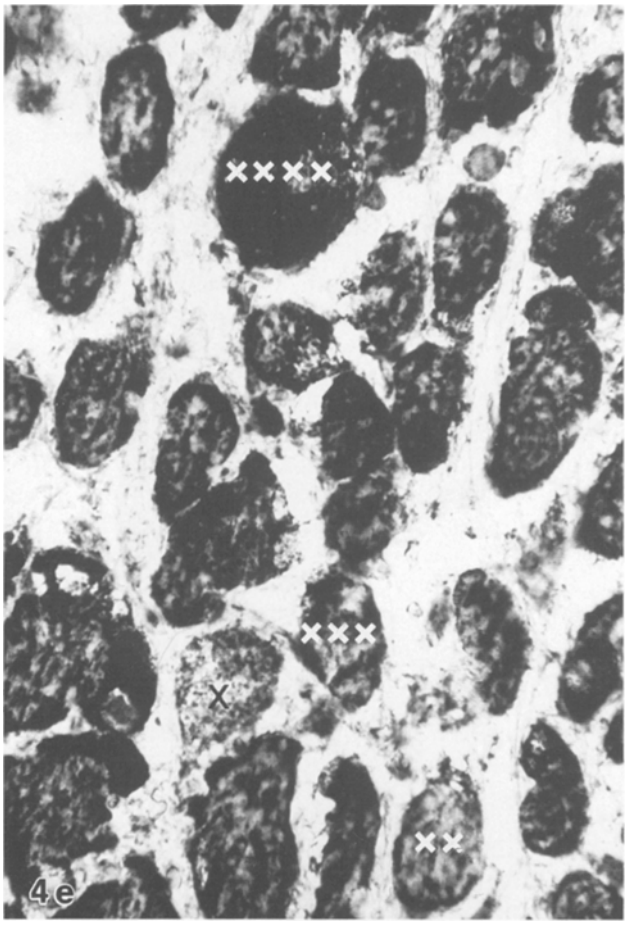


Fig. 3. Superior rectus muscle of a 76-year-old woman lacking hybridization signal with three mtDNA probes (G1, G2, G3), indicating loss of mitochondrial DNA molecules (depletion). **A**, **B** Lack of enzyme protein in three fibres (*x,x,xx*). **A**, mitochondrial subunit II/III. **B**, nuclear subunit IV. **C**, **D**, **E** In situ hybridization of mtDNA with the probes G_1 (C), G_2 (D), G_3 (E) showing no reaction product in one fibre (*XX*). ×640


XXXX
X
XXX
XX
4a
XXXX
X
XXX
XX
4b
XXXX
X
XXX
XX
4c
XXXX
X
XXX
XX
4d



II/III and the nuclear coded subunit IV (Müller-Höcker et al. 1989).

Specific probes of mtDNA (G1, G2, G3, see Fig. 1) were produced by PCR for in situ hybridization. The amplifications were performed in reaction volumes of 100 µl, containing 200 µM each dNTP (dATP, dCTP, dGTP, dTTP), 50 mM potassium chloride, 10 mM TRIS-HCl (pH 8.3), 1.5 mM magnesium chloride, 0.01% gelatine, 30 pmol of each primer and 2.5 units of *Thermus aquaticus* (*Taq*) polymerase (Perkin Elmer Cetus). The primers used for amplification of G1–G3 are located within the human mitochondrial genome according to (Anderson et al. 1981). *G1-For*: nt 4831–4847; *G1-Rev*: nt 5898–5917; *G2-For*: nt 5971–5988; *G2-Rev*: nt 7588–7608; *G3-For*: nt 9265–9282; *G3-Rev*: nt 10088–10107. Human mtDNA, isolated from placenta, served as template DNA in concentrations of 0.1–1.0 ng per amplification reaction (Seibel et al. 1991). The double-stranded DNA obtained was purified by Centricon 100 Microconcentrators (Amicon).

The PCR-DNA fragments were labelled by random primed incorporation (Feinberg and Vogelstein 1983) of digoxigenin-labelled deoxyuridine triphosphate with the digoxigenin labelling kit of Boehringer Mannheim. Purified PCR-DNA was denatured (100° C, 10 min), chilled on ice and mixed with the reaction mixes as described in the protocol of Boehringer Mannheim. To obtain a high amount of synthesized digoxigenin-labelled DNA suitable for in situ hybridizations, the amount of template DNA was adjusted to about 30 ng per labelling reaction. The reaction was finished after 24 h.

The probes G1, G2 will hybridize both with normal mtDNA and mtDNA with the common deletion whereas the probe G3 will only hybridize with normal mtDNA.

In situ hybridization was performed according to Mita et al. (1989) and Shoubridge et al. (1990) with modifications. Frozen sections 8 µm thick were placed on siliconized glass slides, air-dried for 30 min, fixed in 4% paraformaldehyde for 45 min, washed in distilled water, dehydrated in graded alcohol and washed in phosphate-buffered saline (PBS) containing 5 mM magnesium chloride, followed by 10 min proteinase K (5 µg/ml) at room temperature. After a washing step with PBS, acetylation in 0.25% acetic anhydride in 0.1 M triethanolamine was performed at room temperature for 5 min. The sections were then treated with DNase-free RNase (50 µg/ml in 50 mM sodium chloride and 10 µM TRIS/HCl pH 8.00) for 30 min at 37° C and after a washing step transferred into the prehybridization solution (50% formamide/0.6 mM sodium chloride/20 mM TRIS pH 7.5/0.02% Ficoll/0.02% polyvinylpyrrolidone/0.12% bovine serum albumin/1 mM EDTA/0.5 mg/ml salmon sperm DNA (sonicated)/dextran sulphate 10%/0.5 mg/ml total yeast RNA/0.010 µg/ml yeast tRNA) for 2 h at 42° C. Denaturation of the probe and tissue DNA was performed at 92° C. The denatured probe was combined with the prehybridization solution. The slides were hybridized at 42–44° C overnight.

Post hybridization washes were performed, twice in SSC for 1 h at room temperature and in 20% SCC for 3 h at 50° C.

Detection was performed according to the digoxigenin-detection kit of Boehringer Mannheim. Following the development of the reaction the sections were embedded without dehydration or counter staining in glycerol gelatine. Control hybridizations were performed without denaturation of tissue DNA, and with the plasmid probe *pBR328*.

Fig. 4. Different in situ hybridization patterns in the superior rectus muscle of a 79-year-old woman. **A**. Cytochrome c oxidase staining revealing four defective fibres (*x*, *xx*, *xxx*, *xxxx*). **B**. In the succinate dehydrogenase staining all fibres have intensive reaction deposits. **C**. Immunohistochemical detection of subunits II/III. In the defective fibres the enzyme protein is lacking. **D**. In situ hybridization in the common deletion region (G3 probe). In two fibres, (*x*, *xx*) the signal is clearly reduced. In two further fibres (*xxx*, *xxxx*) the signal is normal. **E**. In situ hybridization outside the deletion region (G1 probe). In the fibre *x* a weakened reaction is still seen indicating co-existence of mitochondria with depletion and deletion of mtDNA in the same fibre. The fibre *xx* shows an intensive reaction indicating the presence of the common deletion (see also **Fig. 2**). In the fibres *xxx*, *xxxx*) the hybridization signal is equally intensive. **A–E** × 640

Results

Cytochrome c oxidase staining revealed scattered fibres with loss of enzyme activity (Figs. 2A, 4A). No defects of succinate dehydrogenase (SDH) were seen in the cytochrome c oxidase-deficient fibres (Figs. 2B, 4B). SDH staining was often increased indicating a high content of mitochondria in the cytochrome c oxidase-deficient fibres. Immunohistochemically, loss of immunoreactive enzyme protein was seen in the mitochondrially coded subunits II/III (Figs. 3A, 4C) and in the nuclear coded subunit IV (Fig. 3B), as well as in other nuclear subunits (not shown).

In the cytochrome c oxidase-deficient fibres three different hybridization types existed. In the reaction type A there were single cytochrome c oxidase-deficient fibres with a selective hybridization defect of the probe G3 recognizing the common deletion region (Fig. 2, see methods). Occasionally, in cytochrome c oxidase deficient fibres, no hybridization signal was present with the three probes G1, G2, G3 (Fig. 3) indicating loss of mtDNA molecules (depletion of mtDNA-reaction type

B). Most often the cytochrome c oxidase-deficient fibres showed a normal hybridization pattern with all three probes (reaction type C, Fig. 4). In fibres with the reaction type A or B a small number of normal reacting mitochondria were always present (Fig. 2, see also 4D). Occasionally the co-existence of mitochondria with depletion and of mitochondria with deletion of mtDNA was suggested in single fibres (Fig. 4, E). In longitudinal sections both the enzyme defect and the hybridization defect were segmentally developed and co-localized. No selective hybridization defect was observed for the G2 or G3 probe. No hybridization signals either were obtained with the plasmid probe *pBR328* and without prior denaturation of tissue DNA after RNase treatment. There was no cross-reaction with nuclear DNA.

Discussion

In previous reports dealing with hybridization of the mitochondrial genome in situ radioactive probes have been used (Mita et al. 1989; Shoubridge et al. 1990; Collins et al. 1991). In situ hybridization of mtDNA with digoxigenin as a marker molecule has to our knowledge not yet been reported.

The specificity of the present results is well established; there is no cross-reaction with nuclear DNA in situ and with a plasmid probe *pBR328*. Omitting the heat denaturation step which leads to unfolding of the DNA double helix, also led to negative hybridization results. In addition cross-reaction with RNA could be excluded by pretreatment with DNAse-free RNAse prior to the hybridization step.

The major finding of the present study is that in cytochrome c oxidase-deficient extra-ocular muscle fibres of elderly subjects different hybridization patterns of mtDNA co-exist, a normal hybridization signal with different mtDNA probes including a probe recognizing the common deletion region (position 8,482–13,460 basepairs), a selective hybridization defect in the common deletion region and a reduced or absent signal with three different hybridization probes indicating depletion of mtDNA. No selective hybridization defect was observed with the two probes outside the common deletion region. A systematic in situ screening of mtDNA has, however, not yet been performed.

In previous reports on hybridization of the mitochondrial genome in situ in Kearns-Sayre syndrome accumulation of deleted mtDNA in respiratory deficient fibres has been reported in skeletal muscle (Mita et al. 1989; Shoubridge et al. 1990; Collins et al. 1991). In contrast, during ageing most of the cytochrome c oxidase-deficient extra-ocular muscle fibres showed a normal hybridization signal. These results imply that the common deletion is of minor importance in causing the cytochrome c oxidase defects during ageing because clustering of the deleted mtDNA molecules – a necessary prerequisite for causing the enzyme defect – was not the main alteration in the present study. Therefore it has to be assumed that the mutated mtDNA molecules, which in one study were estimated at 3–9% in the heart of man 80–90 years old (Sugiyama et al. 1991) are more widespread in elderly human muscle than in mitochondrial myopathies.

The reduced hybridization signal with different mtDNA probes recognizing different segments of mtDNA is indicative of depletion of mtDNA. Otherwise one would have to assume different or multiple deletions in the same fibre segment, and the same mitochondrial population. This, however, appears unlikely and has not yet been demonstrated. The possibility that the loss of mtDNA is due to a diminished mitochondrial mass is excluded by the SDH-staining reaction and by the ultracytochemical findings of a normal or increased content of mitochondria in the defective fibres (Müller-Höcker et al. 1992).

The pathophysiological mechanisms leading to such a depletion of mtDNA are unclear. Recently depletion of mtDNA has been reported in early and later onset infantile mitochondrial myopathies expressing either diffuse or focal deficiency of cytochrome c oxidase (Moraes et al. 1991; Tritschler et al. 1992). In these cases with an autosomal inheritance, apparently defective nuclear factors involved in mtDNA replication appear the most likely cause. Recently it has been shown that zidovudine, used in HIV therapy, may also lead to depletion of mtDNA by interfering with mtDNA replication through blockage of gamma DNA polymerase (Arnaudo et al. 1991).

In a case of Kearns-Sayre syndrome, characterized by cardiomyopathy, cardiac cytochrome c oxidase deficiency (Müller-Höcker et al. 1986) and the common deletion of mtDNA (Obermaier-Kusser et al. 1990), we have observed a similar depletion of mtDNA (Müller-Höcker et al. 1992). Therefore the question of pathogenetic interrelations between deletion and depletion of mtDNA arises. This assumption is further suggested by the occasionally observed colocalization of both phenomena in the same fibre (Fig. 4E) as seen in the present study.

Generally it is assumed that the deleted mtDNA molecules have a replication advantage due to their smaller size, thus accumulating during life especially in the nonmitotic skeletal and heart muscle cells, causing progressive impairment of respiratory chain function. This replication advantage, however, may be lost when replication of mtDNA is handicapped due to progressive energy failure caused by the deleted mtDNA molecules. Loss of mtDNA may thus ensue due to a progressive block in the synthesis of mtDNA replication proteins. Furthermore, "dilution" of mtDNA may occur because mitochondrial proliferation apparently may still continue under these conditions, as is seen in the infantile mtDNA depletion syndrome (Moraes et al. 1991; Tritschler et al. 1992) and in zidovudine myopathy (Dalacas et al. 1990), which both feature an increased content of mitochondria. The combined occurrence of mtDNA deletion and depletion has recently been described (Otsuka et al. 1990).

The normal hybridization pattern in most of the cytochrome c oxidase-deficient fibres in the extraocular muscles implies that during ageing depletion and deletion of mtDNA play a minor role in causing cytochrome

c oxidase deficiency. The normal hybridization pattern does not, however, exclude alteration of mtDNA. Point mutations of tRNAs in particular, would be most consistent with defective synthesis of mitochondrial cytochrome c oxidase subunits and have been demonstrated in various mitochondrial diseases (Wallace 1989; Goto et al. 1990; Kobayashi et al. 1990; Shoffner et al. 1990; Enter et al. 1991; Lauber et al. 1991; Obermaier-Kusser et al. 1991; Tanaka et al. 1991). The presence of a minor proportion of deleted mtDNA molecules in those fibres with a normal in situ hybridization signal cannot, however, be excluded. Their pathophysiological importance however, should be negligible. In vitro experiments with cybrids containing deleted and non-deleted mtDNA have shown that an excess of mutated mtDNA is necessary to cause the enzyme defect (Hayashi et al. 1991). Furthermore the loss of cytochrome oxidase protein including both mitochondrial and nuclear subunits of the enzyme in the ageing skeletal muscle (see also Müller-Höcker et al. 1992) points to the involvement of additional (probably nuclear) factors interfering with protein synthesis, import, assembly or degradation in the mitochondria.

Free radical damage of mtDNA and membranes by oxygen radicals has repeatedly been assumed to be an important contributory factor in ageing (Miquel et al. 1980; Fleming et al. 1982; Harman 1983; Nohl 1988; Linnane et al. 1989; Kadenbach and Müller-Höcker 1990; Miquel 1991; Müller-Höcker 1992). This hypothesis is based on: An approximately ten times higher frequency of mutations in mitochondrial as compared to nuclear DNA, the compactness of the genome, increasing the probability that mutations will affect functionally important regions, the lack of adequate repair mechanisms, and the increased production of oxygen radicals in mitochondria with increasing age, promoting chemical modification of mitochondrial proteins and DNA.

The present results indicate that cytochrome c oxidase defects occurring during ageing are associated with different molecular alterations of mtDNA. It is, however, still unclear whether ageing of mitochondria represents a primary factor in the ageing process or merely is a consequence of it. Nevertheless, the decline of respiratory chain function with ageing is an important factor in the progressive decline of functional organ reserve capacity in senescence.

Acknowledgements. This study was supported by grants from the Deutsche Forschungsgemeinschaft (Mu 755/1–3), the Wilhelm-Sander-Stiftung and the Fritz-Thyssen-Stiftung. The authors are indebted to Sabine Dolling and Maria Wittmaier for preparing the manuscript.

References

Anderson S, Bankier AT, Barrell BG, De Bruin MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG (1981) Sequence and organization of the human mitochondrial genome. Nature 290:457–465

Arnaudo E, Dalacas M, Shanske S, Moraes CT, DiMauro S, Schon EA (1991) Depletion of muscle mitochondrial DNA in AIDS patients with zidovudine induced myopathy. Lancet 337:508–510

Attardi G (1981) Organization and expression of the mammalian mitochondrial genome: a lesson in economy. Trends Biochem Sci 6:86–89, 100–103

Attardi G, Schatz G (1988) Biogenesis of mitochondria. Annu Rev Cell Biol 4:289–333

Byrne E, Dennett X, Trounce I, Burdon J (1985) Partial cytochrome oxidase (aa_3) deficiency in chronic progressive external ophthalmoplegia – Histochemical and biochemical studies. J Neurol Sci 71:257–271

Byrne E, Dennett X, Trounce I (1991) Oxidative energy failure in postmitotic cells: a major factor in senescence. Rev Neurol 147:532–535

Chomyn A, Mariottini P, Leeter MWJ, Ragan CI, Doolittle RF, Matsuno-Yagi A, Hatefi Y, Attardi G (1985) Functional assignment of the products of the unassigned reading frames of human mitochondrial DNA. In: Quagliariello E, Slater EC, Palmieri F, Saccone C, Kroon AM (eds) Achievements and perspectives of mitochondrial research. vol II Biogenesis, Elsevier, Amsterdam, pp 259–275

Collins S, Rudduck C, Marzuki S, Dennett X, Byrne E (1991) Mitochondrial genome distribution in histochemically cytochrome c oxidase negative muscle fibres in patients with a mixture of deleted and wild type mitochondrial DNA. Biochem Biophys Acta 1097:309–317

Cormier V, Rötig A, Tardieu M, Colonna M, Saudubray JM, Munnich A (1991) Autosomal dominant deletions of the mitochondrial genome in a case of progressive encephalomyopathy. Am J Hum Genet 48:643–648

Cortopassi GA, Arnheim N (1990) Detection of a specific mitochondrial DNA deletion in tissues of older humans. Nucleic Acids Res 18:6927–6933

Dalacas MC, Illa I, Pezeshkpour, Laukaitis JP, Cohen B, Griffin JL (1990) Mitochondrial myopathy caused by longterm zidovudine therapy. N Engl J Med 322:1098–1105

Degoul F, Nelson I, Lestienne P, Francois D, Romero N, Duboc D, Eymard B, Fardeau M, Ponsot G, Paturneau-Jouas M, Chaussain M, Leroux JP, Marsac C (1991) Deletion of mitochondrial DNA in Kearns-Sayre syndrome and ocular myopathies: genetic biochemical and morphological studies. J Neurol 101:168–177

DiMauro S, Bonilla E, Zeviani M, Nakagawa M, DeVivo DC (1985) Mitochondrial myopathies. Ann Neurol 17:521–538

DiMauro S, Zeviani M, Servidei S, Bonilla E, Miranda AF, Prelle A, Schon EA (1987) Cytochrome c oxidase deficiency: clinical and biochemical heterogeneity. Ann NY Acad Sci 488:19–32

Enter C, Obermaier-Kusser B, Müller-Höcker J, Zierz St, Kurlemann G, Pongratz D, Förster C, Gerbitz KD (1991) A specific point mutation in the mitochondrial genome of Caucasians with MELAS. Hum Genet 88:233–236

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments of high specific activity. Anal Biochem 132:6–13

Fleming JE, Miquel J, Gottroll SF, Yengoyan LS, Economos AC (1982) Is cell ageing caused by respiratory-dependent injury to the mitochondrial genome? Gerontology 28:44–53

Gerbitz KD, Obermaier-Kusser B, Zierz S, Pongratz D, Müller-Höcker J, Lestienne P (1990) Mitochondrial myopathies: divergences of genetic deletions, biochemical defects and the clinical syndromes. J Neurol 237:5–10

Goto Y, Nonoka I, Horai S (1990) A mutation in the tRNA-$^{Leu(UUR)}$gene associated with the MELAS subgroup of mitochondrial encephalopathies. Nature 348:651–653

Hansford RG (1983) Bioenergetics in ageing. Biochem Biophys Acta 726:41–80

Harding AE (1991) Neurological disease and mitochondrial genes. Trends Neurosci 14:132–138

Harman A (1983) Free radical theory of ageing: consequences of mitochondrial ageing. Age 6:86–94

Hattori K, Tanaka M, Sugiyama S, Obayashi T, Ito T, Satake

T, Hanaki Y, Asai Y, Nagano M, Ozawa T (1991) Age dependent increase in deleted mitochondrial DNA in the human heart: possible contributory factor to presbycardia. Am Heart J 121:1735–1742

Hayakawa M, Torii K, Sugiyama S, Tanaka M, Ozawa T (1991) Age-assoziated accumulation of 8-hydroxydeoxyguanosine in mitochondrial DNA of human diaphragm. Biochem Biophys Res Commun 179:1023–1029

Hayashi JI, Ohta S, Kikuchi A, Takemitsu M, Goto Y, Nonaka I (1991) Introduction of disease-related mitochondrial DNA deletions into HeLa cells lacking mitochondrial DNA results in mitochondrial dysfunction. Proc Natl Acad Sci USA 88:10614–10618

Holt IJ, Harding AE, Morgan-Hughes JA (1988) Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. Nature 331:717–719

Ikebe S, Tanaka M, Ohno K, Sato W, Hattori K, Kondo T, Mizuno Y, Ozawa T (1990) Increase of deleted mitochondrial DNA in the striatum in Parkinson's disease and senescence. Biochem Biophys Res Commun 170:1044–1048

Johnson MA, Turnbull DM, Dick DJ, Sherratt HSA (1983) A partial deficiency of cytochrome c oxidase in chronic progressive external ophthalmoplegia. J Neurol Sci 60:31–53

Kadenbach B, Kuhn-Nentwig L, Büge U (1987) Evolution of a regulatory enzyme cytochrome c oxidase (complex IV). Curr Top Bioenerg 15:113–161

Kadenbach B, Müller-Höcker J (1990) Mutations of mitochondrial DNA and human death. Naturwissenschaften 77:221–225

Katayama M, Tanaka M, Yamamoto H, Ohbayashi T, Nimura Y, Ozawa T (1991) Deleted mitochondrial DNA in the skeletal muscle of aged individuals. Biochem Int 25:47–56

Kobayashi Y, Momoi MY, Tominaga K, Momoi T, Nihei K, Yanagisawa M, Kagawa Y, Ohta S (1990) A point mutation in the mitochondrial $tRNA^{Leu}$ (UUR) gene in MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes). Biochem Biophys Res Commun 173:816–822

Larsson NG, Holme E, Kristiansson B, Oldfors A, Tulinius M (1990) Progressive increase of the mutated mitochondrial DNA fraction in Kearns-Sayre syndrome. Pediatr Res 28:131–136

Lauber J, Marsac, Kadenbach B, Seibel P (1991) Mutations in mitochondrial tRNA genes: a frequent cause of neuromuscular diseases. Nucleic Acids Res 19:1393–1397

Lestienne P, Ponsot G (1988) Kearns-Sayre syndrome with muscle mitochondrial DNA deletion. Lancet I:885

Linnane AW, Marzuki S, Ozawa T, Tanaka M (1989) Mitochondrial DNA mutations as an important contributor to ageing and degenerative diseases. Lancet I:642–645

Linnane AW, Baumer A, Maxwell RJ, Preston H, Zhang C, Marzukis (1990) Mitochondrial gene mutation: the ageing process and degenerative disease. Biochem 22:1067–1076

Lojda Z, Gossrau R, Schiebler TH (1976) Enzymhistochemische Methoden. Springer, Berlin Heidelberg New York

Lombes A, Bonilla E, DiMauro S (1989) Mitochondrial encephalomyopathies. Rev Neurol 145:671–689

Miquel J (1991) An integrated theory of aging as the result of mitochondrial DNA mutation in differentiated cells. Arch Gerontol Geriatr 12:99–117

Miquel J, Economos AC, Fleming J, Johnson JE (1980) Mitochondrial role in cell ageing. Exp Gerontol 15:575–591

Mita S, Schmidt B, Schon EA, DiMauro S, Bonilla E (1989) Detection of "deleted" mitochondrial genomes in cytochrome-c-oxidase-deficient muscle fibers of a patient with Kearns-Sayre syndrome. Proc Natl Acad Sci USA 86:9509–9513

Moraes CT, DiMauro S, Zeviani M, Lombes A, Shanske S, Miranda AF, Nakase H, Bonilla E, Werneck LC, Servidei S, Nonaka I, Koga Y, Spiro AJ, Brownell KW, Schmidt B, Schotland DL, Zupanc M, DeVivo DC, Schon EA, Rowland LP (1989) Mitochondrial DNA deletions in progressive external ophthalmoplegia and Kearns-Sayre syndrome. N Engl J Med 320:1293–1299

Moraes CT, Shanske S, Tritschler HJ, Aprille JR, Andreeta F, Bonilla E, Schon EA, DiMauro S (1991) mtDNA depletion with variable tissue expression: a novel abnormality in mitochondrial diseases. Am J Hum Genet 48:492–501

Morgan-Hughes JA, Schapira AHV, Cooper JM, Holt IJ, Harding AE, Clark JB (1990) The molecular pathology of respiratory-chain dysfunction in human mitochondrial myopathies. Biochim Biophys Acta 1018:217–222

Müller-Höcker J (1989) Cytochrome-c-oxidase deficient cardiomyocytes in the human heart. An age-related phenomenon. Am J Pathol 134:1167–1171

Müller-Höcker J (1990) Cytochrome-c-oxidase deficient fibres in the limb muscle and diaphragm of men without muscular disease: an age related alteration. J Neurol Sci 14–21

Müller-Höcker J (1992) Mitochondria and ageing. Brain Pathol 2:149–158

Müller-Höcker J, Schneiderbanger K, Stefani FH, Kadenbach B (1992) Progressive loss of cytochrome-c-oxidase in the extraocular muscles during ageing. A cytochemical-immunocytochemical study. Mutation Res 275:115–124

Müller-Höcker J, Pongratz D, Hübner G (1983) Focal deficiency of cytochrome-c-oxidase in skeletal muscle of patients with progressive external ophthalmoplegia. Virchows Arch (A) 402:61–71

Müller-Höcker J, Stünkel S, Pongratz D, Hübner G (1985) Focal deficiency of cytochrome-c-oxidase and of mitochondrial ATPase with histochemical evidence of loosely coupled oxidative phosphorylation in a mitochondrial myopathy of a patient with bilateral ptosis. An enzyme histochemical, immunohistochemical and fine structural study. J Neurol Sci 69:27–36

Müller-Höcker J, Johannes A, Droste M, Kadenbach B, Pongratz D, Hübner G (1986) Fatal mitochondrial cardiomyopathy in Kearns-Sayre syndrome with deficiency of cytochrome c oxidase in the cardiac and skeletal muscle. An enzymehistochemical-ultraimmunocytochemical fine structural study in longterm frozen autopsy tissue. Virchows Arch [B] 52:353–367

Müller-Höcker J, Droste M, Kadenbach B, Pongratz D, Hübner G (1989) Fatal mitochondrial myopathy with cytochrome-c-oxidase deficiency and subunit restricted reduction of enzyme protein in two siblings. An autopsy-immunocytochemical study. Hum Pathol 20:666–672

Müller-Höcker J, Seibel P, Schneiderbanger K, Zietz C, Obermaier-Kusser B, Gerbitz KD, Kadenbach B (1992) In situ Hybridisation of mitochondrial DNA in the heart of a patient with Kearns-Sayre syndrome and dilatative cardiomyopathy. Hum Pathol (in press)

Nelson I, Degoul F, Obermaier-Kusser B, Romero N, Borrone C, Marsac C, Vayssiére JL, Gerbitz K, Fardeau M, Ponsot G, Lestienne P (1989) Mapping of heteroplasmic mitochondrial DNA deletion in Kearns-Sayre syndrome. Nucleic Acids Res 17:8117–8124

Nohl H (1988) Oxygen radical release in mitochondria: Influence of age. Mod Ageing Res 8:77–99

Obermaier-Kusser B, Müller-Höcker J, Nelson I, Lestienne P, Riedele Th, Gerbitz KD (1990) Detektion of different copy numbers of identically deleted mitochondrial DNA in tissues from a patient with Kearns-Sayre syndrome. Biochem Biophys Res Commun 169:1007–1015

Obermaier-Kusser B, Paetzke-Brunner, Enter C, Müller-Höcker J, Zierz S, Ruitenbeck, Gerbitz KD (1991) Respiratory chain activity in tissues from patients (MELAS) with a point mutation of the mitochondrial genome ($tRNA^{Leu(UUR)}$) FEBS Lett 286:67–70

Otsuka M, Niijima K, Mizuno Y, Yoshida M, Kagawa Y, Ohta S (1990) Marked decrease of mitochondrial DNA with multiple deletions in a patient with familial mitochondrial myopathy. Biochem Biophys Res Com 167:680–685

Petty RKH, Harding AE, Morgan-Hughes JA (1986) The clinical features of mitochondrial myopathy. Brain 109:915–938

Romero NB, Lestienne P, Marsac C, Paturneau-Jouas M, Nelson I, Francois D, Eymard B, Fardeau M (1989) Immunocytological and histochemical correlation in Kearns-Sayre with mtDNA

deletion and partial cytochrome c deficiency in skeletal muscle. J Neurol Sci 93:297–309

Schon EA, Rizzuto R, Moraes CT, Nakase H, Zeviani M, DiMauro S (1989) A direct repeat is a hotspot for large-scale deletion of human mitochondrial DNA. Science 244:346–349

Seibel P, Mell O, Hannemann A, Müller-Höcker J, Kadenbach B (1991) A method for quantitative analysis of deleted mitochondrial DNA by PCR in small tissue samples. Methods Mol Cell Biol 2:147–153

Shanske S, Moraes CT, Lombes A, Miranda AF, Bonilla E, Lewis BA, Whelan HA, Ellsworth CA, DiMauro S (1990) Widespread tissue distribution of mitochondrial DNA deletion in Kearns-Sayre syndrome. Neurology 40:24–28

Shoffner JM, Lott MT, Lezza AMS, Seibel P, Ballinger SW, Wallace DC (1990) Myoclonic epilepsy and ragged-red fibers disease (MERRF) is associated with a mitochondrial DNA $tRNA^{lys}$ mutation. Cell 61:931–937

Shoubridge EA, Karpati G, Hastings KEM (1990) Deletion mutants are functionally dominant over wild-type mitochondrial genomes in skeletal muscle fibre segments in mitochondrial disease. Cell 62:43–49

Sugiyama S, Hattori K, Hayakawa M, Ozawa T (1991) Quantitative analysis of age associated accumulation of mitochondrial DNA with deletion in human heart. Biochem Biophys Res Commun 180:894–899

Tanaka M, Ino H, Ohno K, Ohbayashi T, Ikebe S, Sano T, Ichiki T, Kobayashi M, Wada Y, Ozawa T (1991) Mitochondrial DNA mutations in mitochondrial myopathy, encephalopathy lactic acidosis and stroke-like episodes (MELAS). Biochem Biophys Res Commun 174:861–868

Tritschler HJ, Andreeta F, Moraes CT, Bonilla E, Arnaudo E, Danon MJ, Glass S, Zelaya BM, Vamos E, Shanske S, Kadenbach B, DiMauro S, Schon EA (1992) Mitochondrial myopathy of childhood with depletion of mitochondrial DNA. Neurology 42:209–217

Trounce I, Byrne E, Marzuki S (1989) Decline in skeletal muscle mitochondrial respiratory chain function: possible factor in ageing. Lancet I:637–639

Wallace DC (1989) Mitochondrial DNA mutations and neuromuscular disease. Trends Genet 5:9–13

Yamamoto M, Nonaka I (1988) Skeletal muscle pathology in chronic progressive external ophthalmoplegia with ragged red fibers. Acta Neuropathol 76:558–563

Yen Tch, Su JH, King KL, Wei YH (1991) Ageing associated 5 kb deletion in human liver mitochondrial DNA. Biochem Biophys Res Commun 178:124–131

Yoneda M, Tanno Y, Horai S, Ozawa T, Miyatake T, Tsuji S (1990) A common mitochondrial DNA mutation in the t-RNA^{Lys} of patients with myoclonus epilepsy associated with ragged-ref fibers. Biochem Int 21:789–796

Yuzaki M, Ohkoshi N, Kanazawa I, Kagawa Y, Ohta S (1989) Multiple deletions in mitochondrial DNA at direct repeats of non-D-loop regions in cases of familial mitochondrial myopathy. Biochem Biophys Res Commun 164:1352–1357

Zhang C, Baumer A, Maxwell RJ, Linnane AW, Nagley Ph (1992) Multiple mitochondrial DNA deletions in an elderly human individual. FEBS Lett 297:34–38

Zeviani M, Moraes CT, DiMauro S, Nakase H, Bonilla E, Schon EA, Rowland LP (1988) Deletions of mitochondrial DNA in Kearns-Sayre syndrome. Neurology 38:1339–1346

Zeviani M, Servidei S, Gellera C, Bertini E, DiMauro S, DiDonato S (1989) An autosomal dominant disorder with multiple deletions of mitochondrial DNA starting at the D-loop region. Nature 339:309–311