Detection of “deleted” mitochondrial genomes in cytochrome-c oxidase-deficient muscle fibers of a patient with Kearns–Sayre syndrome

(mitochondrial myopathy/deletion of mitochondrial DNA/in situ hybridization/immunocytochemistry)

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ABSTRACT Using in situ hybridization and immunocytochemistry, we studied a muscle biopsy sample from a patient with Kearns–Sayre syndrome (KSS) who had a deletion of mitochondrial DNA (mtDNA) and partial deficiency of cytochrome-c oxidase (COX; EC 1.9.3.1). We sought a relationship between COX deficiency and abnormalities of mtDNA at the single-fiber level. COX deficiency clearly correlated with a decrease of normal mtDNA and, conversely, deleted mtDNA was more abundant in COX-deficient fibers, especially ragged-red fibers. The distribution of mtRNA had a similar pattern, suggesting that deleted mtDNA is transcribed. Immunocytochemistry showed that the nuclear DNA-encoded subunit IV of COX was present but that the mtDNA-encoded subunit II was markedly diminished in COX-deficient ragged-red fibers. Because the mtDNA deletion in this patient did not comprise the gene encoding COX subunit II, COX deficiency may have resulted from lack of translation of mtRNA encoding all three mtDNA-encoded subunits of COX.

Holt et al. (1) reported deletions of muscle mitochondrial DNA (mtDNA) in 9 of 25 patients with mitochondrial myopathies defined by morphological criteria. Large-scale deletions in muscle mtDNA were then identified by Zeviani et al. (2) in 7 of 7 patients with Kearns–Sayre syndrome (KSS). Those deletions ranged in size from 2.0 to 7.0 kilobases (kb) and did not localize to any specific region of the mtDNA, but six of them comprised at least one of the genes encoding the three large subunits of cytochrome-c oxidase (COX; ferro-cytochrome-c:oxygen oxidoreductase, EC 1.9.3.1) (2).

A consistent and conspicuous finding in muscle biopsy samples from patients with KSS is the presence of a population of fibers lacking histochemically detectable COX (3–7). Because a proportion of these COX-deficient fibers shows no other morphological abnormality of mitochondria, it has been proposed that they may be precursors of ragged-red fibers and that COX deficiency could be a factor in the pathogenesis of KSS and other disorders (3, 8).

We have therefore used in situ hybridization combined with COX histochemistry and immunocytochemistry to explore a possible correlation between alterations in mtDNA and COX deficiency at the single-fiber level.

MATERIALS AND METHODS

Source of Human Muscle. We studied a quadriceps muscle biopsy specimen from one patient (patient 3 from ref. 2) with typical KSS (onset before age 20, ophthalmoplegia, pigmentary retinopathy, and heart block). This patient had also been described by Shy et al. (9) and Berenberg et al. (ref. 10, patient 1). The breakpoint of the mtDNA deletion in the patient was determined at the nucleotide level (11). It was 4977 base pairs (bp) long, extending from nucleotide position 8483 to 13,460 (12), and was flanked (in normal mtDNA) by a 13-bp direct repeat (Fig. 1). The deletion encompassed structural genes for part of ATPase subunit 8; all of ATPase subunit 6, COX subunit III, and NADH-coenzyme Q reductase (ND) subunits 3 and 4; and part of ND5 (11, 12) (Fig. 1). As controls, we used muscle samples from two patients that, after complete neurological evaluation at Columbia-Presbyterian Medical Center, were deemed to be free of neuromuscular disease, and one sample from a possible carrier of Duchenne muscular dystrophy.

Muscle biopsy samples were initially frozen in melting isopentane, sectioned for diagnostic purposes, and stored in liquid nitrogen until the time of this study. Serial transverse 4-μm or 8-μm sections from controls and from the patient were cut from frozen muscle blocks at −18°C with a cryostat, placed onto the same glass slide, and stored at −70°C prior to in situ hybridization and other cytochemical studies. All samples were obtained in accordance with the guidelines of the Columbia University Human Subjects Committee.

Histochemistry. Eight-micrometer-thick sections were utilized for the demonstration of COX and succinate dehydrogenase (SDH) as previously described (13). Additional sections were stained with hematoxylin and eosin and with Gomori trichrome.

Preparation of mtDNA Probes. Specific regions of mtDNA contained in M13 clones (a kind gift of G. Attardi and M. King, California Institute of Technology) were excised from the vectors by digestion with EcoRI plus HindIII (which cut only in the flanking polylinker region) and were then extracted from low-melting agarose gel after electrophoretic separation. The probe designated mt12 contains the 1525-bp Mbo I fragment from mtDNA position 8729 to 10,254 (12) and comprises the genes encoding part of ATPase 6, COX III, tRNAAsp, and a part of the ND3 coding region: this fragment is completely missing in the mtDNA of the patient. Therefore, this probe will hybridize only with normal mtDNA and will be referred to as the ‘‘normal probe.’’ The mt9 probe contains the 846-bp Xba I fragment from positions 7441 to 8287 and covers the genes encoding tRNASer, tRNAAsp, and COX II, which are not deleted in the mtDNA of the patient. Because this probe will hybridize with all mtDNAs, both deleted and undeleted, it will be referred to as the ‘‘common probe’’ (Fig. 1). Plasmid pBR322 was linearized with EcoRI and used as a control probe. All probes were labeled with

Abbreviations: mtDNA, mitochondrial DNA; mtRNA, mitochondrial RNA; KSS, Kearns–Sayre syndrome; COX, cytochrome-c oxidase; SDH, succinate dehydrogenase.

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Localization of the mtDNA deletion in the patient with KSS and of the mtDNA probes employed in the study. Maps just below the scale show the heavy (upper map) and light (lower map) strands of linearized mtDNA, showing the genes for the subunits of NADH-coenzyme Q reductase (ND), COX (CO), and ATP synthase (ATPase), for cytochrome b (CYT B), and for the 12S and 16S ribosomal RNAs. The tRNA genes are denoted by small open boxes. The origins of heavy-strand (OH) and light-strand (OL) replication and the promoters for initiation of heavy-strand (P1H and P2H) and light-strand (Pl) transcription are indicated by arrows. Muscle mtDNA from the patient is represented by a bar. The undeleted region is denoted by the solid areas; the 4977-bp deleted fragment is shown by the open area defined by dashed lines. Portions of mtDNA used as probes are represented by bars under m9 (common probe) and m12 (normal probe). The sequence of the mtDNA in the region of the deletion is shown at the bottom; the direct repeat flanking the deletion (11) is boxed.

**In Situ Hybridization.** We employed published methods with minor modifications (15–17). Eight-micrometer sections from control muscle and from the patient's muscle were placed on the same slide, fixed in freshly prepared 4% paraformaldehyde, and washed twice in phosphate-buffered saline (PBS) containing 5 mM MgCl2. Sections were then treated with protease K (5 μg/ml) for 10 min at room temperature, washed in PBS, and incubated with 0.1 M glycine/0.2 M Tris-HCl, pH 7.5, for 10 min. After washing in PBS, the samples were treated with RNase A (50 μg/ml) at 37°C for 30 min, if indicated, and then placed for 1 hr in prehybridization solution [50% formamide/600 mM NaCl/10 mM Tris-HCl, pH 7.5/1 mM EDTA/0.12% polyvinylpyrroli- done/0.12% Ficoll/0.6% bovine serum albumin (BSA)] with denatured salmon sperm DNA (200 μg/ml), total yeast RNA (0.5 mg/ml), and yeast tRNA (50 μg/ml). The sections were then placed in 70% formamide/2× SSC (0.3 M NaCl/0.03 M sodium citrate, pH 7.0) for 5 min at 75°C to denature cellular DNAs. The denatured probe was combined with prehybridization solution containing 10% dextran sulfate and 10 mM dithiothreitol so that the final probe activity was 106 cpm/μl of hybridization solution. The sections were overlaid with the hybridization solution and incubated at 42°C for 18 hr. In situ hybridization to mtDNA, but not mtRNA, was performed after RNase A treatment; for mtRNA, hybridization was performed in samples that were not subjected to DNA-denaturing conditions. For the simultaneous detection of hybridization and COX activity, sections were first stained for COX (13) and then treated according to the protocol for in situ hybridization to mtDNA. To demonstrate the validity of the results, control hybridizations were performed with pBR322 probe, or with m9 or m12 probe after RNase A treatment without subjecting the samples to DNA-denaturing conditions.

After hybridization, the samples were rinsed in 2× SSC for 1 hr at room temperature and in 0.1× SSC for 3 hr at 50°C, air-dried, exposed to x-ray film for 3–5 days, dipped in NTB2 emulsion (Kodak), and stored in the dark for 7–14 days. Finally, the samples were developed, fixed, rinsed in water, and counterstained with hematoxylin and eosin to determine the cytological localization of the hybridization signal. The background signal was determined by subtracting the number of grains noted in regions outside of the sections from those observed over the sections in the samples processed as control hybridizations.

**Immunocytochemistry.** We used polyclonal antibodies raised in rabbits against a synthetic peptide comprising 11 amino acids at the C terminus of the mitochondrially encoded subunit II of COX (kindly provided by Russell F. Doolittle, University of California at San Diego, La Jolla). In immunocytochemistry of unfixed frozen muscle sections, these antibodies stained only mitochondria, and in Western blots of mitochondrial preparations, they recognized only subunit II of COX (18, 19). We also employed a monoclonal antibody directed against the nuclear-encoded subunit IV of COX. This antibody stained mitochondria and recognized only subunit IV in Western blots of mitochondrial preparations (19). For these studies, unfixed 4-μm-thick sections from the...
patient’s muscle and from a control were placed on the same coverslip and incubated for 2 hr at room temperature with the polyclonal or monoclonal antibodies (20–25 μg/ml) in PBS containing 1% BSA. Control sections were incubated with nonimmune serum or with PBS alone. The sections were washed three times in PBS and incubated for 1 hr at room temperature with fluorescein-conjugated goat anti-rabbit IgG or anti-mouse IgG (30–40 μg/ml) in PBS containing 1% BSA. The samples were washed three times in PBS and mounted in PBS containing 50% (vol/vol) glycerol (20).

All sections were examined and photographed with a Zeiss II photomicroscope equipped with epi-illumination.

RESULTS

Histochemistry. The incidence of muscle fibers with little or no COX activity was determined in samples from four different fascicles of the biopsy specimen. Serial sections stained for SDH activity were used to determine the incidence of fibers showing abnormal accumulations of mitochondria or ragged-red fibers. The proportion of COX-deficient fibers was 51%, and the proportion of ragged-red fibers was 7.7%. All fibers with ragged-red appearance were COX-deficient.

In Situ Hybridization. In the patient with KSS, the intensity of the hybridization signal for either mtDNA or mtRNA was greatly decreased with the mt12 probe (Fig. 1), which detected only normal mtDNA and/or mtRNA (Fig. 2 A and B). With the mt9 (common) probe (Fig. 1), which detected both normal and deleted mtDNA and/or mtRNA, the intensity of the hybridization signal for either mtDNA or mtRNA was higher in the patient with KSS than in the control sample (Fig. 2 C and D). Comparison of the signal intensities obtained with the two probes (Fig. 2, compare A to C and B to D) implies that the majority of the signal in the patient’s muscle arising from the common probe derives from hybridization with DNA or RNA from the deleted genomes. Sections hybridized with control pBR322 probe did not show any signal (Fig. 2E).

By light microscopy, high grain density was seen in normal muscle fibers. The concentration of mtDNA was higher in type I than in type II fibers and the distribution of grains was homogeneous within any given fiber (Fig. 3 A and B). In the patient, normal mtDNA—that is, DNA hybridizing with the normal probe—was present at high abundance in fibers with COX activity, at moderate abundance in fibers with partial COX deficiency, and at lowest abundance in fibers lacking COX activity (Fig. 3C). Also, the distribution of normal mtDNA was homogeneous in fibers with COX activity but heterogeneous in those lacking COX activity. In COX-deficient fibers, the grains were concentrated at the subsarcolemmal region and were lacking or sparse at the center of the fibers (Fig. 3 C and G). The distribution of normal mtRNA agreed closely with that of the normal mtDNA (Fig. 3D).

![Fig. 3: COX histochemistry and localization of mtDNA and mtRNA with the mt12 (normal) probe in control muscle (A and B) and in samples from the patient with KSS (C–G). (A) COX histochemistry shows type I and type II fibers (white circles). (B) In situ hybridization in adjacent serial section for the detection of mtDNA shows grains in all muscle fibers; grains are less abundant in type II fibers (white circles). (C) In situ hybridization combined with COX histochemistry shows high grain density in fibers with COX activity (white circles). Fewer grains (black stars) are noted in fibers with partial COX activity and even fewer in fibers lacking COX activity (black circles); the latter show reduced grain density at the center and more grains at the cell periphery (arrow). (D) In situ hybridization in an adjacent serial section for the detection of mtRNA shows that the localization and distribution of mtRNA are similar to those of the mtDNA. (E–G) In situ hybridization combined with COX histochemistry for detection of mtDNA. The serial sections show one muscle fiber (+) at different levels of cross-sectioning. Note the correlation between COX activity and normal mtDNA; in E the fiber shows COX activity and numerous grains; in F the fiber shows lower COX activity and fewer grains; in G the fiber lacks COX activity and there are only a few grains at the center of the fiber, but grains appeared concentrated at the cell periphery (arrows). (A and B, ×200; C and D, ×160; E–G, ×260.)](image-url)
In sections of the patient's muscle, COX activity and grain density in single fibers varied widely in serial sections hybridized with the normal probe, suggesting that COX deficiency and distribution of normal mtDNA in those fibers were segmental (Fig. 3 E–G).

With the common probe, the hybridization signal was high in all muscle fibers. The grains were much more abundant in COX-deficient ragged-red fibers identified by SDH staining. The grains were also more abundant in non-ragged-red fibers lacking COX activity than in fibers with COX activity (Fig. 4 A and C). The distribution of mtRNA showed a pattern similar to that of the mtDNA, suggesting that mtRNA putatively transcribed from deleted mtDNA was much more abundant in COX-deficient ragged-red fibers (Fig. 4B).

The specificity of the hybridization experiments was confirmed by the observation that only a few grains above background were detected in sections hybridized with the pBR322 probe and in sections hybridized with mtDNA probes after RNase A treatment without subjecting the samples to DNA-denaturing conditions (data not shown).

**Immunocytochemistry.** Immunostaining of patient's muscle was always carried out in parallel with control muscle, and an adjacent section was used for the enzymatic demonstration of COX activity.

**COX subunit IV.** Normal muscle showed discrete particulate (mitochondrial) immunoreactivity that was more prominent in type I fibers (data not shown). In the patient's sample, all fibers showed immunoreactivity and this was especially intense in the subsarcolemmal region of COX-deficient ragged-red fibers (Fig. 5 A and B).

**COX subunit II.** In normal muscle there was discrete particulate immunostain of mitochondria that was more prominent in type I fibers (data not shown). In the patient's sample, there was virtual absence of reaction at the center of COX-deficient ragged-red fibers, with discrete deposits of immunoreactivity at the cell periphery. Particulate immunostain was seen in fibers with COX activity (Fig. 5 A and C). Control sections incubated with nonimmune serum showed no immunostain.

**DISCUSSION**

This patient had the essential clinical manifestations of KSS, as well as morphological and biochemical evidence of mitochondrial myopathy. A small proportion of fibers were "ragged-red" with the Gomori trichrome stain; electron microscopy showed abnormal mitochondria with crystalline inclusions; and biochemical studies demonstrated a partial defect of COX activity (2, 10, 21, 22). Southern blot analysis of muscle mtDNA showed a large deletion of almost 5 kb that included the gene encoding subunit III of COX (2, 11).

COX is a complex enzyme that comprises 13 subunits. The three largest peptides (I–III), which are encoded by mtDNA and are synthesized within the mitochondria, confer the catalytic and proton-pumping activities on the enzyme. The 10 smaller subunits are synthesized in the cytoplasm under the control of nuclear genes and are presumed to confer tissue specificity, thus adjusting the enzymatic activity to the metabolic demands of different tissues (23). In our patient, several fibers showed either no or markedly reduced enzyme activity by COX histochemistry. All ragged-red fibers lacked COX activity, but COX deficiency was also found in a significant proportion of fibers without abnormal proliferation of mitochondria. These observations support previous
suggestions that COX deficiency may precede the appearance of morphologic changes of mitochondria (3, 8).

In situ hybridization in the patient's muscle showed that normal and deleted mtDNAs have distinct patterns of cellular localization. Undeleted mtDNA was present in fibers with COX activity but was reduced in fibers with COX deficiency. On the other hand, deleted mtDNA was more abundant in COX-deficient non-ragged-red fibers, and was also present in COX-deficient non-ragged-red fibers. The focal accumulation of deleted mtDNA implies that replication has occurred: this is possible because the origins of heavy- and light-strand replication were preserved in the deleted mtDNA of the patient (2, 11).

Our data also suggest that the deleted mtDNA is transcriptionally active and that the message is more abundant in COX-deficient ragged-red fibers. Due to the lack of indispensable tRNAs, translation should not be possible in mitochondria containing only deleted mtDNA, but translation could occur in mitochondria containing both normal and deleted mtDNAs, due to intraorganelle complementation. Bogenhagen and Clayton (24) reported that mitochondria in HeLa cells contain from 2 to 10 copies of mtDNA, and complementation has been documented in cultured cells by Watson and co-workers (25). However, the lack of COX enzymatic activity in ragged-red fibers implies lack of effective complementation, at least at the level of sensitivity of the histochemical stain and at the level of resolution provided by the light microscope. Biochemical analysis of individual cellular clones from another KSS patient supports this contention (26). Studies of COX activity at the electron microscopic level in COX-deficient fibers of KSS should help clarify this issue.

Immunocytochemical studies with antibodies directed against the mitochondrially-encoded subunit II of COX showed markedly decreased immunoreactivity in COX-deficient ragged-red fibers, while other fibers stained positively. Immunocytochemistry with antibodies directed against the nuclear-encoded COX IV showed that all fibers, including COX-deficient ragged-red fibers, stained positively. The decrease of COX II in ragged-red fibers containing mitochondria with a deletion of mtDNA that did not involve the gene for subunit II supports the concept that the entire mitochondrial genome functions as a single genetic unit rather than as a series of individual genes (25, 27). The transcription of mtDNA is polystrionic; that is, all the genes of the heavy and light strands are transcribed as two large RNAs, which are later cleaved into individual tRNAs and mRNAs (25, 28–30). A deletion anywhere in the genome could affect transcription and translation, even of genes that are not directly encompassed by the deletion. Our in situ hybridization evidence that the deleted mtDNA is transcriptionally active further suggests that COX deficiency may result from lack of translation of mtRNA encoding the three larger subunits of COX. While we cannot exclude that COX II protein might be synthesized and rapidly degraded, the presence of COX IV immunoreactivity in COX-deficient ragged-red fibers suggests that unassembled COX subunits may not be degraded very rapidly.

COX deficiency in muscle of patients with KSS is segmental and progressive, affecting an increasing proportion of fibers during the course of the disease (7). The segmental loss of COX activity could be due to a progressive increase in the proportion of deleted mtDNA, probably resulting from a clonal expansion of a single deletion event (2, 11). Such apparent replicative advantage of deleted mtDNA may be due to the fact that the smaller DNA size allows for faster replication (31), but this remains to be documented. This possibility could be explored further by applying the tech-