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Immunohistochemical detection of human mtDNA polymerase gamma and of human mitochondrial transcription factor A in cytochrome-c-oxidase-deficient oxyphil cells of hyperfunctional parathyroids

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Abstract Immunohistochemical studies were performed in 18 hyperfunctional parathyroids with oxyphil cell aggregates for the detection of cytochrome-c-oxidase (complex IV of the respiratory chain), mitochondrial DNA polymerase gamma and human mitochondrial transcription factor A (h-mtTFA). Seventy-three oxyphil areas exhibiting a defect of cytochrome-c-oxidase were found. The defect involved both the mitochondrially coded subunits II/III and the nuclear derived subunits Vab. There was no loss of mtDNA polymerase gamma or of h-mtTFA in these foci, corresponding to a high content of mtDNA revealed by in situ hybridization. Isolated defects of h-mtTFA were also not found. In contrast, isolated defects of mtDNA polymerase gamma were present in 22 oxyphil foci. These results show that defects of cytochrome-c-oxidase in oxyphil cells are not due to altered expression of h-mtTFA or DNA polymerase gamma, indicating that other nuclear factors involved in the generation of the respiratory chain may be impaired. The low incidence of defects of mtDNA polymerase gamma and the absence of alterations of h-mtTFA and cytochrome-c-oxidase in these foci suggest that defects of mtDNA polymerase gamma are of minor pathogenetic significance.

Key words Cytochrome-c-oxidase · Mitochondrial DNA · In situ hybridization · mtDNA polymerase gamma · Human mitochondrial transcription factor A

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Introduction

Defects of the respiratory chain are a characteristic feature of mitochondrial diseases [6, 10, 11, 23, 37, 43] but also occur as a normal phenomenon during ageing in various organs [27], including the liver [33] and the brain [15]. In recent years molecular genetic studies have shown that mutations of mitochondrial DNA (mtDNA) such as deletions, duplications and point mutations in both transfer RNAs (tRNA) and in structural genes are involved in the pathogenesis of mitochondrial diseases [4, 12, 19, 43, 45, 49, 50]). Similar molecular genetic alterations are also seen during ageing, although in milder forms [13].

In recent studies we have shown that the mitochondria-rich oxyphil cells of the parathyroid [28, 32] and the liver [29], and of the thyroid in Hashimoto thyroiditis [34], are especially prone to developing defects of cytochrome-c-oxidase (complex IV). Molecular genetic analysis of mtDNA revealed no consistent abnormalities to clarify the pathogenesis of these defects. Since the respiratory chain is composed of proteins derived from both the nuclear and the mitochondrial genome, defects of nuclear genes must also be considered. In fact, about 90% of the proteins in the respiratory chain are derived from the nucleus, while the mitochondrial genome codes for only 13 proteins. Furthermore, replication and transcription of mtDNA are completely under the control of nucleus-derived factors [3, 7, 44]. Human mitochondrial transcription factor A (h-mtTFA) is considered to be an important regulator of both transcription and replication of mtDNA [7, 14, 21, 26, 36, 44]. In fact, it has been shown that h-mtTFA may be deficient in a subset of mitochondrial diseases [20, 38]. Mitochondrial dysfunction may also arise from inhibition of mtDNA polymerase gamma, the only DNA polymerase involved in replication of the mitochondrial genome [39]. Inhibition of DNA polymerase gamma by chemical compounds such as zidovudine and others have been shown to lead to depletion of mitochondrial DNA and may induce typical mitochondrial myopathies [22].

In the present study the coordinated expression of cytochrome-c-oxidase, h-mtTFA, mtDNA polymerase gamma and in situ hybridization of mtDNA has been studied in oxyphil cells of the parathyroid glands. The results indicate that faulty expression of h-mtTFA or mtDNA polymerase gamma is not involved in the pathogenesis of cytochrome-c-oxidase defects in oxyphil cells of the parathyroids. There is, however, a minor subset of oxyphil foci with deficient expression of mtDNA polymerase gamma.

Materials and methods

The investigations were carried out on 18 parathyroid glands of patients with hyperparathyroidism. Hyperplasia of parathyroids was present in 15 cases. In 3 an adenoma was diagnosed. Hyperplasia of the parathyroids was diagnosed when multiglandular nodular or diffuse enlargement (gland size larger than 6×4×2 mm) was present, often accompanied by a reduced content of interstitial fat tissue.The tissue was fixed routinely in formalin and embedded in paraffin for further studies.

Immunohistochemistry was performed for the detection of cytochrome-c-oxidase. Cytochrome-c-oxidase is composed of 13 subunits, the three largest (I-III) of which are encoded by mtDNA. These three subunits are essential for enzyme function, because they contain the catalytic centre. The nuclear subunits probably have regulatory functions and are responsible for organ-specific isoenzyme expression [17]. Immunohistochemistry was performed on paraffin-embedded tissue with subunit-specific polyclonal antisera raised in rabbits against the mitochondrially derived subunits II/III and nuclear subunit Vab of cytochrome-c-oxidase as previously described [30]. The antisera were kindly provided by Prof. B. Kadenbach (Fachbereich Biochemie, Philipps-Universität Marburg).

Oligonucleotide primers specific to the human DNA polymerase gamma-cDNA were used to amplify the polymerase domain of the human gene [39]. The amplified products were subcloned into the *E. coli* expression vector pGEX2T (Pharmacia, Uppsala, Sweden) to express the DNA polymerase gamma peptide, G714 to L1061, as a glutathione-*S*-transferase fusion protein (CD-7). The CD-7 antigen was expressed and purified from *E. coli* DH5 alpha. Anti-CD7 antibodies were raised in rabbits and purified over protein-A agarose.

Western blots of nuclear, mitochondrial and cytoplasmic extracts of Hela cells probed with the specific antibody revealed a 140-kDa protein in the mitochondrial extract and no cross-reacting proteins in the nuclear or cytosolic extracts. A clone for human h-mtTFA was established by reversed transcription PCR and subcloned into *E. coli* [26]. The purified protein was used for raising antibodies in rabbits. Characterization was carried out on Western blots.

Specific probes of mtDNA (G1, G2, G3; Fig. 1) were produced by a polymerase chain reaction (PCR) for in situ hybridization. The amplifications were performed in reaction volumes of 100 µL containing 200 µmol/l each of deoxyadenosine triphosphate (dATP); deoxycytidosine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP), 50 mmol/l potassium chloride; 10 mmol/l TRIS-HCl (pH 8.3), 1.5 mmol/l magnesium chloride; 0.01% gelatin; 30 pmol of each primer; and 2.5 U thermus aquaticus (Taq) polymerase (Perkin Elmer Cetus, Überlingen, Germany). The primers used for amplification of G1–G3 are located within the human mitochondrial genome [35]; G1-For, np 4,831–4,847; G1-Rev, np 5,898–5,917; G2-For, np 5,971–5,988; G2-Rev, np 7,588–7,608; G3-For, np 9,265–9,282; and G3-Rev, np 10,088–10,107 (Fig. 1). Human mtDNA isolated from placenta served as template DNA in concentrations of 0.1–1.0 ng per amplification reaction [40]. The double-stranded DNA obtained was purified by Centricon 100 Microconcentrators (Amicon, Beverly, Mass.).

The PCR-DNA fragments were labelled by random primed incorporation of digoxigenin (DIG)-labelled deoxyuridine triphosphate using the DIG labelling kit from Boehringer Mannheim (Mannheim, Germany). Purified PCR-DNA was denatured (100°C for 10 min), chilled on ice, and mixed with the reaction mixes as described in the protocol (Boehringer Mannheim). To obtain a large amount of synthesized DIG-labelled DNA suitable for in situ hybridization, the amount of template DNA was adjusted to approximately 30 ng per labelling reaction. The reaction was finished after 24 h. The probes G1 and G2 will hybridize both with normal mtDNA and with mtDNA containing the common deletion (np 9.482–13.459), whereas probe G3 will only hybridize with normal mtDNA (Fig. 1).

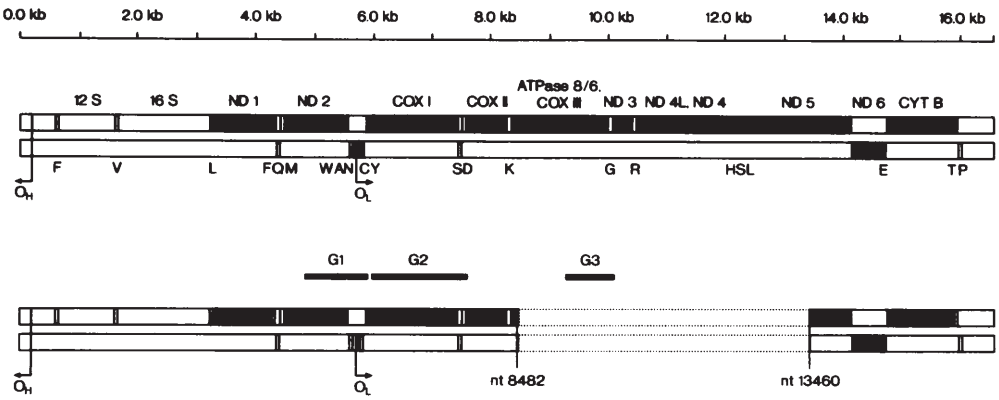
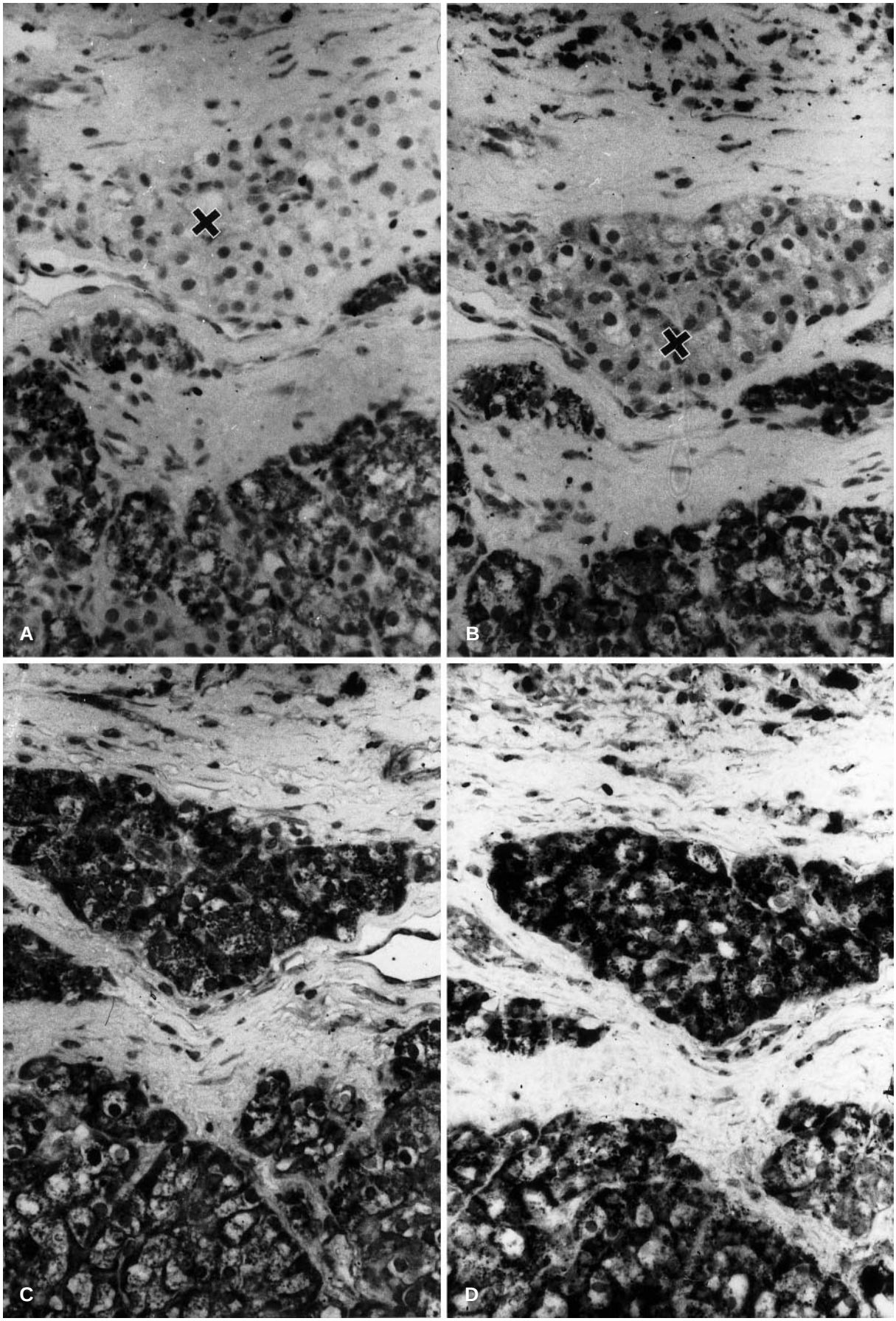


Fig. 1 Linearized map of mtDNA with localization of mtDNA probes G1, G2 and G3 and of the 4.977 base pair deletion ("common deletion") at nucleotide position 8,482 on the *left side* and 13,460 on the *right side* of the mitochondrial genome. The following genes are indicated by *boxes*: *ND 1-6* and *ND 4L* of nicotinamide adenine dinucleotide dehydrogenase (complex 1), cytochrome B (*CYT B*, complex 3), cytochrome-c-oxidase (subunits 1–3, complex 4), ATP 6, ATP 8 of ATP synthase (complex 5), and large (*16S*) and small (*12S*) ribosomal RNA. O_H and O_L indicate the origins of replication of the heavy and light strands, respectively. The tRNA genes are not shown

Fig. 2A–D Immunohistochemistry of cytochrome-c-oxidase. **A** Subunit II/III; **B** subunit Vab; **C** mtDNA polymerase gamma; **D** human mitochondrial transcription factor A (h-mtTFA). There are oxyphil cells with high reactivity for subunit II/III and Vab (**A**, **B**) An oxyphil foci lacking immunoreactive cox subunits (*x*) is shown. mtDNA polymerase gamma and h-mtTFA are highly expressed in both oxyphil foci. ×640 ▶


A
B
C
D

In situ hybridization was performed according to Mita et al. [25] and Shoubridge et al. [46], with modifications on paraffin-embedded tissue as previously described [31]. Sections 8 µm thick were placed on siliconized glass slides, then air-dried for 30 min. Deparaffination was performed as usual with xylol for 30 min, followed by rehydration. The slides were then washed in phosphate-buffered saline containing 5 mmol/l magnesium chloride, followed by a 10-min wash in proteinase K (5 µm/ml) at room temperature. After another wash with phosphate-buffered saline, acetylation in 0.25% acetic anhydride in 0.1 mol/l triethanolamine was performed at room temperature for 5 min. The sections were then treated with DNAse-free RNAse (50 µg/ml in 50 mmol/l NaCl and 10 µmol/l TRIS/HCl, pH 8.00) for 30 min at 37°C and, after washing, transferred into the prehybridization solution (50% formamide, 0.6 mmol/l NaCl, 20 mmol/l TRIS (pH 7.5), 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.12% bovine serum albumin, 1 mmol/l ethylenadiaminetetraacetic acid, 0.5 mg/ml salmon sperm DNA (sonicated), 10% dextran sulphate, 0.5 mg/ml total yeast RNA, and 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°C to 44°C overnight. Posthybridization washes consisted of 2× sodium chloride sodium citrate (SSC) for 1 h at room temperature and 0.2× SSC for 3 h at 50°C.

Detection was performed with the digoxigenin detection kit (Boehringer Mannheim). After the development of the reaction, the sections were embedded without dehydration or counterstaining in glycerol gelatin. Control hybridizations were performed without denaturation of tissue DNA and with the plasmid probe pBR 328.

In situ hybridization was performed on 6 cases.

Results

As previously described [28] normal chief cells and oxyphil cells of the parathyroids were well stained by subunit-specific antisera against the mitochondrially coded subunits II/III and the nuclear derived subunit Vab of cytochrome-c-oxidase (complex IV, Fig. 2]. In addition there were randomly distributed defects of cytochrome-c-oxidase in oxyphil cell aggregates, with loss of both subunits (Figs. 2A, 2B, 3A, 4A) in all cases.

A total of 73 defects were localized in the 18 cases involved in this study. The number of defects varied between 1 and 19 defects/case.

Immunohistochemical detection of mtDNA polymerase gamma and h-mtTFA revealed an identical reaction pattern of varying intensities. The highest staining intensities were found in oxyphil cells (Fig. 2C, D), whereas normal chief cells showed a low to moderate reaction intensity. In all oxyphil foci with defective cytochrome-c-oxidase, mtDNA polymerase gamma and h-mtTFA were regularly expressed and showed an intensive reactivity (Fig. 2C, D, 3B). No selective defects were found for h-mtTFA. In contrast occasional selective defects of mtDNA polymerase gamma occurred (Fig. 4B) with cytochrome-c-oxidase and h-mtTFA being unchanged in this foci (Fig. 4A, C). In all, 22 such defective foci were found, being restricted to 3 cases.

In situ hybridization of mtDNA provided an intensive signal in oxyphil areas with a defect of cytochrome-c-oxidase (Fig. 3C). Oxyphil foci with a depletion of mtDNA were not found. No degenerative changes or inflammatory infiltrates were present. The oxyphil cells defective of cytochrome-c-oxidase or of mtDNA polymerase gamma were cytologically unremarkable. In particular, there was no evidence of cell loss on routine histology.

Discussion

The present investigation substantiates previous studies on the occurrence of defects of the respiratory chain in oxyphil cells of the parathyroid glands [28, 32]. These cells are characterized by a high content of mitochondria and increase with age and in secondary hyperparathyroidism [1, 47].

The mechanisms involved in oxyphil cell transformation are still unknown. Oxyphil cells of the parathyroid may, however, be functionally active [5, 47]. Most interestingly, parathyroid hormone-related protein, the major factor responsible for humoral hypercalcaemia of malignancy, was localized predominantly in oxyphil cells [18]. There is some evidence that oxyphil cells in secondary hyperplasia have lower concentrations of cytoplasmic Ca^{2+} and respond less to incremental changes in extracellular Ca^{2+} than chief cells [16].

Our previous analysis on mutations of mtDNA in oxyphilic defects of cytochrome-c-oxidase did not reveal consistent abnormalities [32]. The common 4,977-base-pair deletion (8.483–13.460) was not found. However, in a recent study revealing defects of cytochrome-c-oxidase in the oxyphil Askanazy cells of Hashimoto thyroiditis an accumulation of the common 4,977-bp deletion was demonstrated exclusively in this type of cell [34].

In this study involving 18 parathyroid glands with hyperfunction and multiple oxyphilic nodules, 73 oxyphilic foci defective in cytochrome-c-oxidase were found and were correlated with the expression of human h-mtTFA and mtDNA polymerase gamma.

Studies on normal parathyroid glands were not included in the present investigation, since a previous study had shown a similar reaction pattern of cytochrome-c-oxidase, the density of cytochrome-c-oxidase defects being, however, lower than in hyperfunctional parathyroids [32].

Immunohistochemical studies of h-mtTFA and mtDNA polymerase gamma have rarely been performed [8, 20, 21]. Human mtTFA plays a major part in both replication and transcription of mitochondrial DNA and has been found to be deficient in a subset of mitochondrial myopathies with depletion of mitochondrial DNA [20, 38]. In cell lines devoid of mtDNA the level of h-mtTFA also was found to be low [20, 38]. In contrast, high levels of h-mtTFA were present in typical ragged red fibres, with a high content of mitochondria and defects of cytochrome-c-oxidase associated with a deletion of mtDNA [20].

The findings in the present study further support these results. The oxyphilic foci deficient of cytochrome-c-oxidase had a high immunohistochemical level of h-mtTFA and of mtDNA as revealed by in situ hybridization.

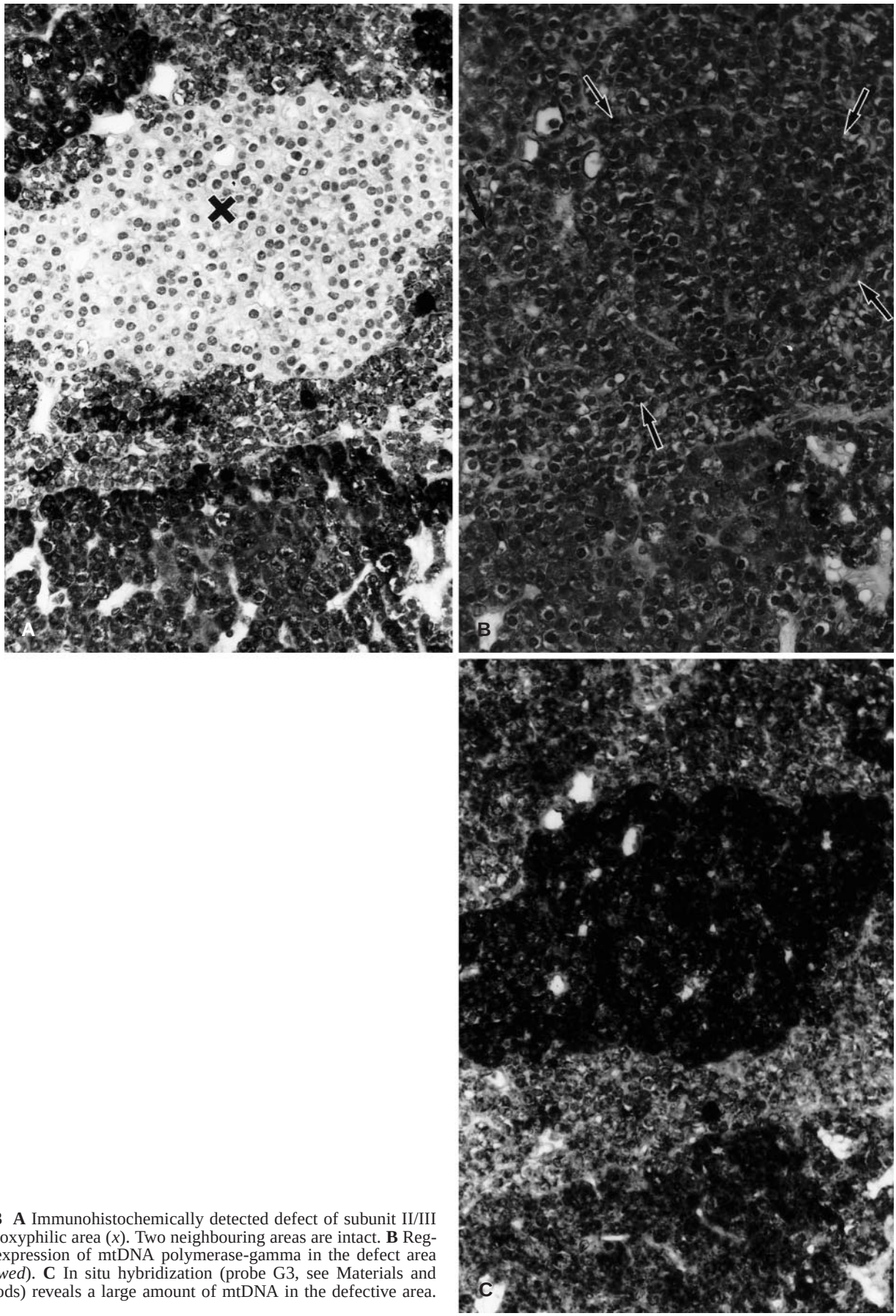


Fig. 3 **A** Immunohistochemically detected defect of subunit II/III in an oxyphilic area (*x*). Two neighbouring areas are intact. **B** Regular expression of mtDNA polymerase-gamma in the defect area (*arrowed*). **C** In situ hybridization (probe G3, see Materials and methods) reveals a large amount of mtDNA in the defective area. ×350

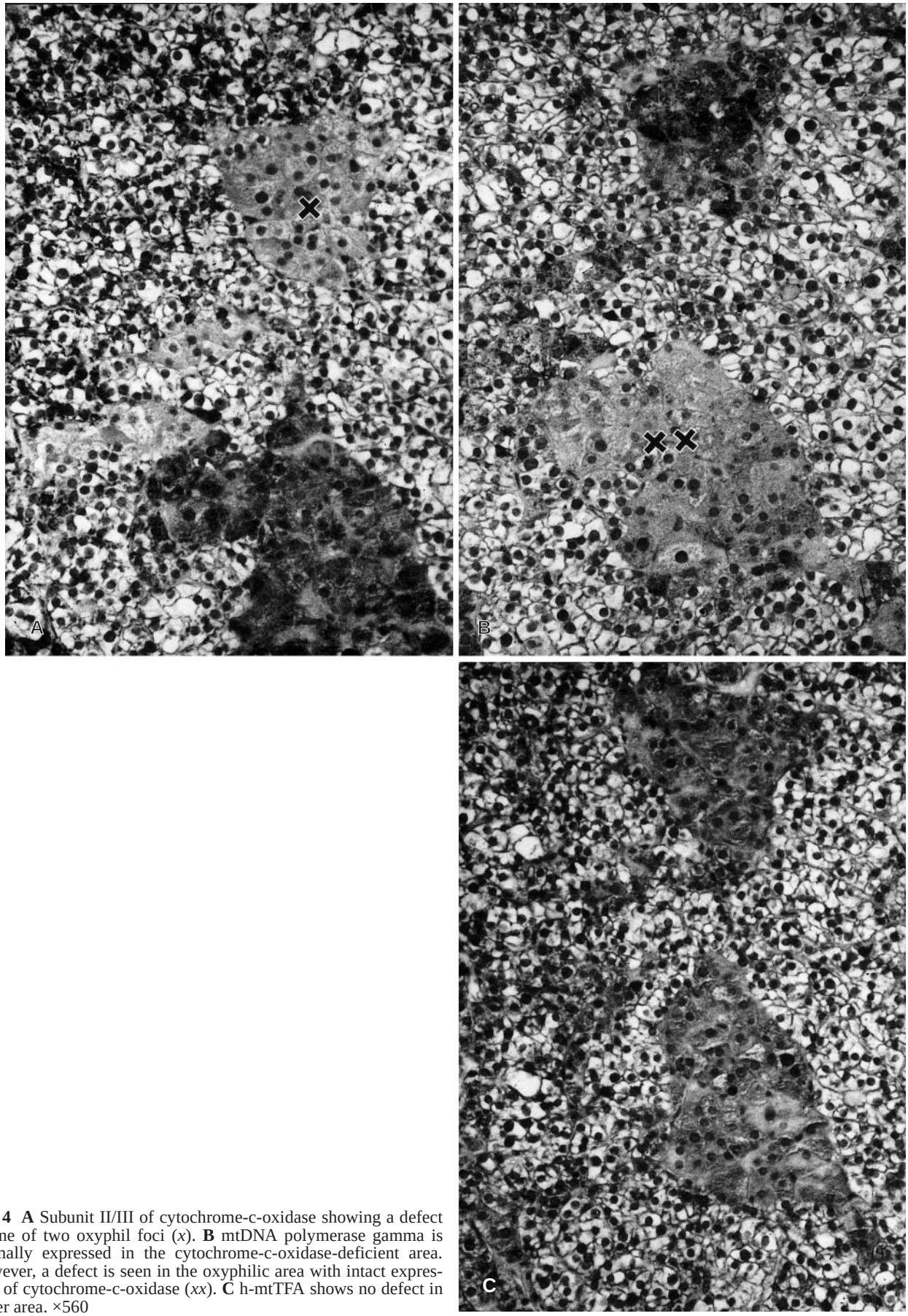


Fig. 4 **A** Subunit II/III of cytochrome-c-oxidase showing a defect in one of two oxyphil foci (*x*). **B** mtDNA polymerase gamma is normally expressed in the cytochrome-c-oxidase-deficient area. However, a defect is seen in the oxyphilic area with intact expression of cytochrome-c-oxidase (*xx*). **C** h-mtTFA shows no defect in either area. ×560

There was no evidence of h-mtTFA deficiency in oxyphil cells with or without defects of complex IV. It is well known that h-mtTFA closely mirrors the mtDNA status of the cell [20, 38].

mtDNA polymerase gamma has been shown to be detected immunocytochemically in cell cultures [9]. Our study provides further evidence that antigenicity is also well preserved after paraffin embedding. No tight linkage of mtDNA polymerase gamma with mtDNA content has been found, in contrast to what is known for h-mtTFA [9]. In the cell culture study mitochondrial DNA polymerase gamma was found to be expressed and translated even in the absence of mitochondrial DNA; thus, mtDNA polymerase gamma is not regarded as a major regulator of mtDNA copy number [9].

In our study we found a low/lacking signal for mtDNA polymerase gamma in a small number of oxyphilic foci. To our knowledge, defects of mtDNA polymerase gamma have so far not been reported. The significance of this finding, however, remains unclear. There was no associated defect either of cytochrome-c-oxidase or of h-mtTFA in these areas. Both findings indicate that replication and transcription of mtDNA seem pretty well intact. This finding is in agreement with the assumption that regulation of copy number of mtDNA is not of primary importance for the transcription of mtDNA [35, 51], the more so as only a small number of mtDNA molecules are transcribed at any given time point within a cell [51].

An artificial cause for the decreased immunoreactivity appears unlikely, considering the circumscribed selective character of the defects. Defective recognition of the protein by the applied polyclonal antibody appears rather unlikely. Decreased synthesis or increased degradation of an abnormal protein is more likely. The generally high immunoreactivity for cytochrome-c-oxidase (except in those areas with defects), h-mtTFA and mtDNA polymerase gamma in oxyphils fits in well with the high content of mitochondria in these cells. Overexpression of these proteins in oxyphil cells as a correlate for the high immunoreactivity appears less likely.

The present study has shown that faulty expression of h-mtTFA and mtDNA polymerase gamma are not involved in the pathogenesis of the defects of cytochrome-c-oxidase in oxyphil cell nodules of the parathyroids. Nuclear factors may also be important in the loss not only of mitochondrial but also of nuclear subunits of cytochrome-c-oxidase. In yeast, for instance, it is well known that more than 30 nuclear factors are necessary for the correct biogenesis and assembly of the enzyme [24]. Among these nuclear transcriptional activators of respiratory factors, NRF 1 and 2 are most promising candidates, since they activate the transcription of three nuclear subunits of cytochrome-c-oxidase, subunits IV, Vb, and Vlc, among other proteins [48]. Unfortunately, antisera against these factors (kindly provided by R.S. Scarpulla) did not prove to be reactive on formalin-fixed paraffin-embedded tissue. The unravelling of the molecular defects in mitochondrial diseases of nuclear origin [41] remains a major challenge. The pathogenetic importance of the defects described here for mtDNA polymerase gamma awaits further clarification.

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