

# An Autosomal Locus Predisposing to Multiple Deletions of mtDNA on Chromosome 3p

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## Summary

Autosomal dominant progressive external ophthalmoplegia (adPEO) is a disorder characterized by ptosis, progressive weakness of the external eye muscles, and general muscle weakness. The patients have multiple deletions of mtDNA on Southern blots or in PCR analysis of muscle DNA and a mild deficiency of one or more respiratory-chain enzymes carrying mtDNA-encoded subunits. The pattern of inheritance indicates a nuclear gene defect predisposing to secondary mtDNA deletions. Recently, in one Finnish family, we assigned an adPEO locus to chromosome 10q 23.3-24.3 but also excluded linkage to this same locus in two Italian adPEO families with a phenotype closely resembling the Finnish one. We applied a random mapping approach to informative non-10q-linked Italian families to assign the second locus for adPEO and found strong evidence for linkage on chromosome 3p 14.1-21.2 in three Italian families, with a maximum two-point lod score of 4.62 at a recombination fraction of .0. However, in three additional families, linkage to the same chromosomal region was clearly absent, indicating further genetic complexity of the adPEO trait.

## Introduction

The respiratory-chain deficiencies are a genetically complex group of disorders, since the enzymes involved are

encoded partially by the nuclear genes and partially by mtDNA. Thus, a gene defect in a nuclear-encoded subunit results in a disease with Mendelian inheritance, whereas a point mutation in mtDNA causes a maternally inherited disorder. Furthermore, two known examples represent involvement of both the genomes: a defect in a nuclear-encoded protein may result in a dramatic reduction in the amount of mtDNA (Moraes et al. 1991) or accumulation of multiple deletions in mtDNA (Zeviani et al. 1989). These disorders are inherited autosomally, and the respiratory-chain defect is most likely a result of the secondary de novo generated mtDNA defects.

We have recently assigned to 10q 23.3-24.3 one nuclear gene locus predisposing to mtDNA mutations in a large Finnish family suffering from autosomal dominant progressive external ophthalmoplegia (adPEO), with maximal lod score of 4.52 at a recombination fraction ( $\theta$ ) of .0 with marker D10S597 (Suomalainen et al. 1995). AdPEO is characterized by progressive weakness of the ocular muscles, ptosis, and fatigue of the skeletal muscles. In addition, bilateral cataract, ataxia, hearing loss, early death, and depression have been reported (Zeviani et al. 1990; Servidei et al. 1991; Suomalainen et al. 1992). Two Italian families were, however, shown not to share the same gene locus with the Finnish family, indicating heterogeneity of the trait (Suomalainen et al. 1995). To localize the second adPEO disease locus we analyzed polymorphic DNA markers in the Italian families. Here we present the assignment of the locus to chromosome 3p 14.1-21.2 in three Italian families and show evidence for yet another locus (loci) predisposing to mtDNA deletions.

## Subjects

Figures 1 and 2 show the pedigrees of the adPEO families. The diagnosis of adPEO was based on marked clinical symptoms or the presence of multiple mtDNA deletions either on Southern blots or in PCR analyses

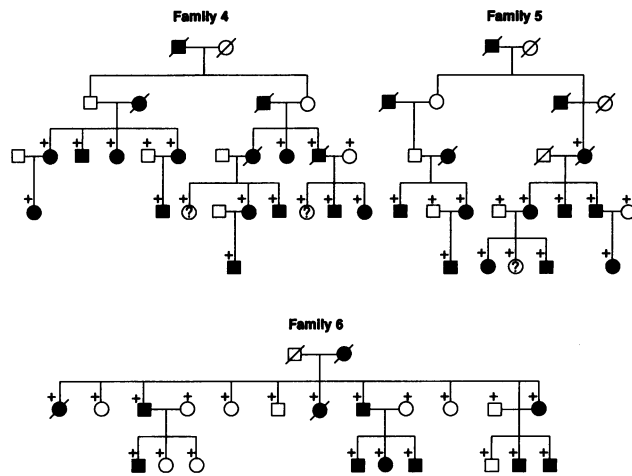
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**Figure 2** adPEO families not linked with markers on chromosome 3p. A plus sign (+) denotes subjects from whom DNA samples were available.

of muscle-biopsy specimens. Italian family 1 has been described in detail by Servidei et al. (1991). The patients had bilateral ptosis with PEO and exercise intolerance, with the exception of individual 304. Dysphagia, dysphonia, and weakness of facial, neck flexor, limb, and respiratory muscles were common to these patients. Individual 304, a 26-year-old man, showed only mild weakness of finger extensors, but the audiogram revealed a mild sensorineural hearing loss, the electromyogram was characterized by moderate myopathic abnormalities, the respiratory-function tests were abnormal, and multiple deletions of muscle mtDNA were detected by PCR. The age at onset of symptoms ranged from 24 to 30 years in this family. Cataract was a later symptom, common to affected individuals >40 years of age. High levels of blood lactic acid were observed after standard exercise, in several affected individuals. Examination of five muscle biopsies revealed the presence of variable amounts of ragged-red and cytochrome *c* oxidase (COX)-negative fibers, and partial reduction of COX-specific activity. The presence of multiple deletions of muscle mtDNA was confirmed by Southern blotting or PCR, in five patients from this family. The symptoms of the adPEO patients in families 2–5 resembled those of family 1. However, in families 2 and 3, the age at disease onset was ~40 and 35 years, respectively, and

the neuromuscular symptoms were largely confined to eye and facial muscles in family 2. Clinically healthy individual 301 (42 years old) from this family was considered affected, because the muscle-biopsy specimen revealed lipid accumulation, scattered COX-negative fibers, and fiber atrophy, and the Southern blot analysis of muscle mtDNA showed the presence of multiple mtDNA deletions. Individual 302 (40 years old) of this family was considered unaffected, because the muscle biopsy was morphologically normal, no muscle mtDNA rearrangements were detected by PCR or Southern blot analyses, and he was clinically healthy. In family 3, a 70-year-old clinically normal man (person 203) was also considered unaffected because he had seven clinically unaffected offspring. Subject 206 (70 years old) of this family was clinically healthy, and no muscle mtDNA deletions were revealed by Southern blot or PCR analyses. He was, however, considered to be an obligatory gene carrier, because a paternity test confirmed that he was the father of an affected individual (person 312). The clinically healthy individual 311 (44 years) was also considered to be an obligatory gene carrier, because her son (person 403) suffered from grand mal epilepsy, his visual and brainstem evoked potentials were abnormal, and multiple mtDNA deletions of muscle were detected by PCR. Person 404 had grand mal epilepsy as the only clinical symptom. Finnish family 6 has been described elsewhere and shown to be linked to markers on chromosome 10q 23.3-24.3 (Suomalainen et al. 1995). Families 3 and 4 originate from northeastern Italy, and families 1, 2, and 5 each from different parts of Italy.

## Methods

### Extraction of Total DNA and mtDNA Analysis

Total DNA was extracted from lymphoblasts, cultured fibroblasts, or 10–150 mg of frozen muscle by using the method of Davis et al. (1986, pp. 47–50), modified as described by Zeviani et al. (1988). The Southern blot analysis with *Pvu*II restriction digestion of total DNA and the preparation of total human mtDNA as the hybridization probe were performed as described by Zeviani et al. (1988). PCR amplifications were performed using a pair of mtDNA primers from 8200–8225 (sense) to 13530–13555 (antisense) and from 7440–7465 (sense) to 16130–16155 (antisense). Amplifications were performed on 1–5 ng of total DNA.

**Figure 1** adPEO families linked with the markers on chromosome 3p. Blackened symbols indicate individuals with severe PEO, multiple mtDNA deletions on Southern blots or in PCR analysis. Gray symbols indicate obligatory gene carriers (persons 206 and 311, family 3). Open symbols with question marks indicate clinically asymptomatic subjects from whom a muscle-biopsy specimen was not available. Open symbols indicate healthy individuals. Beneath each symbol is shown the individual haplotype covering a 22-cM region on chromosome 3p 14.1-21.2. The corresponding markers are listed beside the haplotypes of family 1. The areas within boxes indicate the shared regions on the affected chromosomes within each family. Subject 304 from family 3 (indicated with an asterisk [\*]) had a double recombination within the region between markers D3S1289 and D3S1547.

The first denaturation was at 96°C for 120 s, followed by 30 cycles, each composed of a denaturation at 94°C for 90 s, annealing at 58°C for 90 s, and extension at 72°C for 150 s. The final extension was at 72°C for 5 min. The Gene-Amp kit (Perkin Elmer-Cetus) was used in all the experiments.

### Genotyping

The primers or the sequences for the polymorphic dinucleotide repeat markers were obtained from the Généthon collection of amplifiable DNA markers (Gyapay et al. 1994) or the Nordic Genome Resource Centre (Uppsala, Sweden). Twelve nanograms of template DNA was amplified in microtiter wells in a volume of 15  $\mu$ l with each primer at 0.4  $\mu$ M and each dNTP at 0.2 mM concentrations, in 10 mM TrisHCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 0.1% Triton X-100, and using 0.25 U DNA polymerase F-501S (Dynazyme, Finnzymes). One of the primers was 5'-labeled with <sup>32</sup>P- $\gamma$ ATP by T4-polynucleotidekinase (Pharmacia). The number of cycles used was 29, each cycle consisting of denaturation at 92°C for 30 s and at the primer-specific annealing temperature for 30 s and extension at 72°C for 30 s, with the exception of the first denaturation at 92°C for 3 min and the final extension at 72°C for 5 min, on a programmable thermal controller (PTC; MJ Research). The radioactive amplified fragments were separated in a 5% denaturing polyacrylamide gel and visualized by autoradiography on Kodak X-Omat films.

### Linkage Analyses

The inheritance pattern in all the families was consistent with an autosomal dominant pattern, and the frequency of the disease gene in the Italian and Finnish populations was estimated to be 1:100,000. The genetic intermarker distances and the order of the markers on 3p were based on published information (Gyapay et al. 1994). All the members of Finnish family 6 had undergone muscle biopsy, as described elsewhere (Suomalainen et al. 1992, 1995). Several asymptomatic subjects of this family had been shown to have multiple mtDNA deletions in their muscle and thus carry the disease gene (Suomalainen et al. 1995). Since muscle samples were not available from most Italian asymptomatic subjects, we included in the linkage analyses only the patients with severe PEO, those subjects with affected offspring and subjects whose muscle-biopsy specimen was available for mtDNA analysis. Persons 302 in family 2 and 203 in family 3 were considered unaffected with 90% disease penetrance. Two obligatory gene carriers in family 3 (persons 206 and 311) were considered affected.

The data-simulation analyses were carried out separately for each family by the SLINK and MSIM options of the LINKAGE package computer program, version 4.91 (Ott 1989; Weeks et al. 1990). The average ex-

pected lod scores from 2,500 generated replicates with a four-allelic simulated marker locus in families 1-6 at  $\theta = .01$  were as follows: family 1, 0.58, SD 0.50; family 2, 0.50, SD 0.50; family 3, 1.49, SD 0.92; family 4, 1.63, SD 0.70; family 5, 1.43, SD 0.71; and family 6, 2.30, SD 1.34. The maximum expected lod scores for the same families at  $\theta = .01$  were 1.45, 1.03, 2.62, 2.75, 2.24, and 4.20, respectively. The linkage analyses were carried out using the FASTLINK option (Cottingham et al. 1993; Schaffer et al. 1994) of the MLINK and LINKMAP programs (version 5.1) of the LINKAGE package (Lathrop et al. 1984). The test for heterogeneity was performed using the multipoint lod scores for each family as an input file for the HOMOG program (version 3.35) (Ott 1991, pp. 203-216). The step size for increments in  $\alpha$  (the proportion of families segregating with the linked gene) was set at 0.01.

Markers D10S198, D10S1264, D10S192, D10S534, D10S543, D10S597, D10S1269, and D10S562 were used to exclude (multipoint lod score  $< -2$  or exclusion by haplotype analyses) linkage in families 1-5 to 10q 23.3-24.3, the region that was closely linked with the disease gene in family 6 (Suomalainen et al. 1995).

## Results

### Random Search for the adPEO Locus

We applied a random search approach throughout the genome, using highly polymorphic dinucleotide repeat markers in the genotype analyses of family 3, selected on the basis of high expected lod scores obtained in simulation analyses. An  $\sim 20$ -cM marker map throughout the genome was constructed. Families 1, 2, 4, 5, and 6 were analyzed after finding positive lod scores in family 3. Although the statistical power of the two-point analysis of the affected-only material was not optimal, the multipoint analysis proved to be efficient for exclusion of the majority of chromosomal regions.

### Assignment of the adPEO Locus

After the analysis of 227 DNA markers, primary evidence for linkage was obtained with marker D3S1300 in family 3, giving a two-point lod score of 2.67 at  $\theta = .0$ , which is close to the expected maximum lod score in this family. When the complete family material was tested for this marker, families 1, 2, and 6 showed positive two-point lod scores (respectively, 1.20, 0.75, and 1.84 at  $\theta = .0$ ), but families 4 and 5 were clearly negative (tables 1 and 2). Markers D3S1312, D3S1600, D3S1285, D3S1547, D3S1295, D3S1606, D3S1581, and D3S1289 were thereafter analyzed. When analyzing all the families combined in multipoint calculations under the assumption of homogeneity, no evidence for linkage was obtained. However, linkage was evident when the possibility for locus heterogeneity was allowed

**Table 1**

**Pairwise Lod Scores of Chromosome 3p Markers with Linked Families**

MARKER	LOD SCORE (Z) AT $\theta =$				
	.00	.01	.05	.10	.15
<b>D3S1289:</b>					
Family 1	-3.93	-.95	-.33	-.12	-.03
Family 2	.15	.15	.16	.16	.14
Family 3	<u>2.54</u>	<u>2.50</u>	<u>2.31</u>	<u>2.06</u>	<u>1.80</u>
Combined	-1.24	1.70	2.14	2.10	1.91
<b>D3S1581:</b>					
Family 1	.35	.34	.29	.24	.18
Family 2	1.05	1.03	.93	.81	.68
Family 3	<u>-∞</u>	<u>.45</u>	<u>.98</u>	<u>1.06</u>	<u>1.01</u>
Combined	<u>-∞</u>	1.82	2.20	2.11	1.87
<b>D3S1606:</b>					
Family 1	.39	.38	.33	.27	.21
Family 2	.61	.59	.52	.43	.34
Family 3	<u>.89</u>	<u>.87</u>	<u>.79</u>	<u>.69</u>	<u>.59</u>
Combined	1.89	1.84	1.64	1.39	1.14
<b>D3S1295:</b>					
Family 1	.96	.94	.84	.71	.58
Family 2	.15	.15	.13	.10	.08
Family 3	<u>.00</u>	<u>.00</u>	<u>.01</u>	<u>.02</u>	<u>.02</u>
Combined	1.11	1.09	.98	.83	.68
<b>D3S1547:</b>					
Family 1	1.33	1.30	1.18	1.03	.87
Family 2	.94	.92	.83	.72	.61
Family 3	<u>.19</u>	<u>.18</u>	<u>.13</u>	<u>.09</u>	<u>.07</u>
Combined	2.46	2.40	2.14	1.84	1.55
<b>D3S1300:</b>					
Family 1	1.20	1.17	1.05	.89	.73
Family 2	.75	.74	.66	.56	.47
Family 3	<u>2.67</u>	<u>2.62</u>	<u>2.43</u>	<u>2.17</u>	<u>1.90</u>
Combined	4.62	4.53	4.14	3.62	3.10
<b>D3S1312:</b>					
Family 1	.78	.76	.66	.54	.43
Family 2	.49	.47	.41	.34	.27
Family 3	<u>2.08</u>	<u>2.04</u>	<u>1.85</u>	<u>1.62</u>	<u>1.37</u>
Combined	3.35	3.27	2.92	2.50	2.07
<b>D3S1600:</b>					
Family 1	-4.12	-1.00	-.37	-.15	-.06
Family 2	.27	.26	.24	.20	.16
Family 3	<u>2.49</u>	<u>2.45</u>	<u>2.26</u>	<u>2.02</u>	<u>1.76</u>
Combined	-1.36	1.71	2.13	2.07	1.86
<b>D3S1285:</b>					
Family 1	-4.39	-1.39	-.71	-.43	-.28
Family 2	-4.22	-.93	-.30	-.08	.01
Family 3	<u>.86</u>	<u>.84</u>	<u>.77</u>	<u>.68</u>	<u>.59</u>
Combined	-7.75	-1.48	-.24	.17	.32

(fig. 3). When testing the data from multipoint analyses by using the HOMOG program, the highest likelihood ratio of linkage given heterogeneity divided by the null hypothesis of no linkage was 5260:1, and a lod score >3.0 in multipoint analyses was observed with values of  $\alpha$  ranging from 0.15 to 0.82 between markers D3S1581 and D3S1600, giving strong statistical evi-

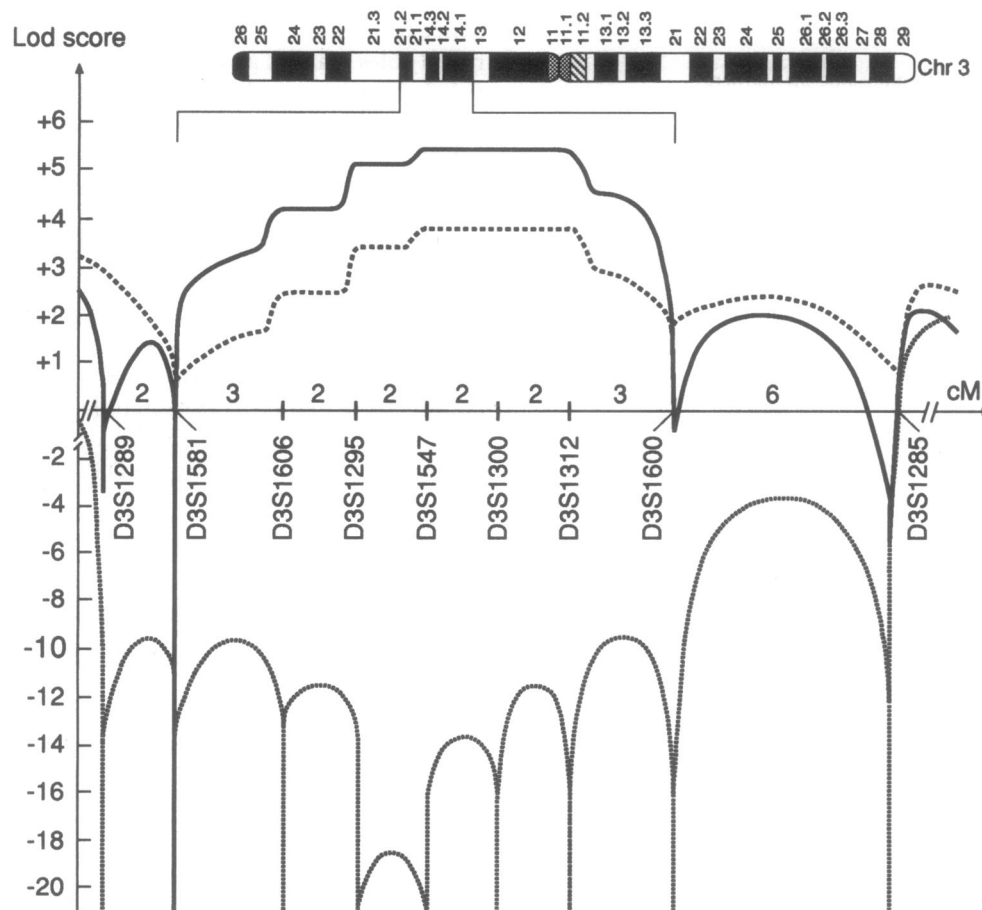
dence for linkage and heterogeneity in this family material. Tables 1 and 2 give the pairwise lod scores between the 3p markers and the adPEO trait. The maximum two-point lod score combined for families 1, 2, and 3 was 4.62 at  $\theta = .0$  with marker D3S1300. The exclusion of families 4, 5, and 6 was based on the haplotype analyses revealing many obligate recombinations throughout the region of interest (data not shown) and multipoint linkage calculations for these families, which showed clear exclusion over the critical region (fig. 3).

All the affected members within families 1, 2, and 3 shared a common chromosomal region of 8 cM extending from marker D3S1606 to D3S1312 (fig. 1). Subject 304 in family 3 harbored a double recombination within a 9-cM region, between D3S1289 and D3S1547.

**Table 2**

**Pairwise Lod Scores of Chromosome 3p Markers with Families Not Linked to 3p Locus**

MARKER	LOD SCORE (Z) AT $\theta =$				
	.00	.01	.05	.10	.15
<b>D3S1289:</b>					
Family 4	-∞	-1.23	-.02	.33	.43
Family 5	1.70	1.65	1.47	1.24	1.01
Family 6	-∞	-5.70	-2.83	-1.61	-9.96
<b>D3S1581:</b>					
Family 4	-∞	-1.10	.10	.45	.54
Family 5	-∞	-1.54	-.29	.14	.31
Family 6	-∞	-.27	.33	.49	.52
<b>D3S1606:</b>					
Family 4	1.83	1.79	1.64	1.43	1.22
Family 5	-∞	.00	.56	.69	.68
Family 6	.59	.57	.52	.45	.38
<b>D3S1295:</b>					
Family 4	-∞	-1.08	-.45	-.22	-.12
Family 5	-4.48	-.70	-.10	.07	.12
Family 6	-∞	-2.84	-1.91	-1.38	-1.00
<b>D3S1547:</b>					
Family 4	-∞	-1.55	-.33	.04	.16
Family 5	1.14	1.12	1.01	.88	.76
Family 6	-∞	-4.15	-2.06	-1.22	-.77
<b>D3S1300:</b>					
Family 4	-∞	-1.47	-.26	.11	.23
Family 5	-∞	-3.01	-1.06	-.35	-.01
Family 6	1.84	1.84	1.77	1.63	1.46
<b>D3S1312:</b>					
Family 4	-∞	-.77	-.17	.00	.05
Family 5	-∞	-2.27	-.97	-.50	-.27
Family 6	-∞	-1.05	.25	.67	.81
<b>D3S1600:</b>					
Family 4	-∞	.23	.73	.78	.72
Family 5	-∞	-2.04	-.78	-.34	-.15
Family 6	-∞	-.75	.53	.93	1.04
<b>D3S1285:</b>					
Family 4	-∞	.30	.83	.91	.86
Family 5	-4.89	-1.21	-.56	-.32	-.21
Family 6	-7.94	-1.94	-.59	-.08	.14



**Figure 3** Multipoint linkage analysis between the adPEO locus and markers on chromosome 3p. The dotted line indicates the multipoint lod scores combined for all the families, on the assumption of locus heterogeneity. The solid line represents the corresponding combined lod scores for families 1-3, and the fine dotted line represents the combined lod scores for families 4-6. The individual DNA markers are shown below the x-axis, and the intermarker distances are shown above the x-axis.

In family 1, subjects 302 and 303 had recombinations between D3S1289 and D3S1581 (fig. 1), and subject 201 had a recombination between markers D3S1312 and D3S1600. These data place the adPEO locus within a 14-cM region between markers D3S1581 and D3S1600 on 3p 14.1-21.2. The cytogenetic localization of the recombination markers was based on data obtained from the Genome Data Base.

### Discussion

Here we report the assignment of the gene causing adPEO in three Italian families to chromosome 3p 14.1-21.2 (Genome Data Base). This second adPEO locus lies within a 14-cM region between markers D3S1581 and D3S1600. All the affected members within these families shared the haplotype flanked by markers D3S1312 and D3S1606. We have earlier identified one adPEO locus on 10q 23.3-24.3, and, among six adPEO families analyzed here, two revealed exclusion for both the 3p and

10q loci. No clear correlation could be found between the disease phenotype and the chromosomal assignment of the disease. Ptosis and ophthalmoplegia were common to all families. Affected individuals of the 10q-linked family had a muscle disease and occasionally avoidant personality or depression (Suomalainen et al. 1992). The psychiatric abnormalities were not encountered in the Italian families. In one 3p-linked family some subjects suffered from grand mal epilepsy, and in another 3p-linked family one patient had a hearing defect, but these features were not found in other 3p-linked families. In an adPEO family not linked to either loci some affected individuals had ataxia, which was absent from the 10q- and 3p-linked families.

In family 3 the penetrance seemed to be age dependent and incomplete. One asymptomatic subject carried the affected haplotype and had an elevated blood lactate level but did not have any apparent deletions of mtDNA detectable in Southern blot analysis of muscle DNA. This may be due to incomplete penetrance or the young

age (24 years) of the subject at the time of the biopsy, since we have previously shown that at least in the 10q-linked adPEO the amount of mutant mtDNA increases with age (Suomalainen et al. 1995). The average age at disease onset in this family was ~35 years. The pattern of inheritance was clearly autosomal dominant, but the penetrance was incomplete in one branch of the family, since a father and her daughter transmitted the disease without being clinically affected.

The observed genetic heterogeneity of adPEO indicates that defects in at least three different nuclear genes predispose to deletions of mtDNA. Either independent functional molecules or an enzyme consisting of several subunits, each encoded in different chromosomal regions, could be involved. The mechanism by which the mtDNA deletions arise has been suggested to be homologous recombination between the molecules or a defect in mtDNA replication (Schon et al. 1989; Shoffner et al. 1989). The deletions could also result from mtDNA damage secondary to the formation of highly reactive oxygen radicals in the respiratory chain. The latter is supported by the fact that the mutant mtDNA is most abundant in tissues most dependent on oxidative metabolism (Suomalainen et al. 1992). Furthermore, the tissue distribution of mutant mtDNA in adPEO patients is the same as in normal aging people, in whose tissues small amounts of mutant mtDNA accumulate during aging, most likely because of cumulative oxidative damage (Cortopassi and Arnheim 1990; Corral-Debrinski et al. 1992). The gene defects underlying adPEO still remain unknown. The cloning of the first adPEO gene may provide new tools for understanding how the two separate genomes communicate and how the defects in this communication might result in human disease.

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