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Functional and Structural Features of a Tandem Duplication of the Human mtDNA Promoter Region

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Summary

An ~260-bp tandem duplication of the human mtDNA regulatory region has been identified in patients with mitochondrial disorders and in a specific Caucasian haplogroup. The functional significance of this mtDNA duplication was difficult to assess, because it was present at very low levels in human tissues. We have isolated several transmitochondrial cybrid lines harboring this mutation, one of which (clone CA17.1) was essentially homoplasmic for the duplication. Oxidative-phosphorylation function was not impaired in clone CA17.1, suggesting that this mtDNA alteration is not pathogenic. mtDNA copy number and steady-state levels of heavy- and light-strand transcripts were unaltered in clone CA 17.1. The steady-state levels of RNAs made from the two promoters (either from the heavy-strand or from the light-strand) were also similar, indicating that oppositely oriented promoters did not interfere with each other.

Introduction

Several mutations in the mtDNA have been associated with human diseases (Wallace 1992; DiMauro and Moraes 1993; Larsson and Clayton 1995). Patients with different clinical manifestations can harbor either single nucleotide substitutions or large-scale rearrangements of mtDNA. One of the most common clinical features associated with large-scale rearrangements (deletions or duplications) of mtDNA is a chronic myopathy with progressive external ophthalmoplegia (PEO) and mitochondrial proliferation in muscle fibers (which are referred to as "ragged-red fibers" [RRF]; Moraes et al. 1989). Duplications of specific mtDNA regions sometimes coexist with large deletions (Rötig et al. 1992; Poulton et al. 1993), but their pathogenetic role is still controversial (Ballinger et al. 1994; Poulton and Holt 1994).

Brockington et al. (1993) suggested that a large fraction of patients with large-scale mtDNA deletions also have a heteroplasmic small tandem duplication in the mtDNA regulatory region. In their study, 50% of the patient's unaffected mothers, who did not harbor mtDNA deletions, also had low levels of the small duplication. The duplicated region was an ~260-bp fragment, starting at a polycytosine stretch (nucleotides 302–308; numbered as in Anderson et al. 1981) and ending at another polycytosine stretch (nucleotides 567–573). mtDNAs with this small tandem duplication (hereafter referred to as "260dup mtDNA") were present in muscle or blood at very low abundance and, in most cases, could be detected only by PCR. Brockington et al. (1993) suggested that this particular duplication could be a predisposing factor for the genesis of mtDNA deletions. Further work by Manfredi et al. (1995) could not confirm the prevalence of this small duplication in patients with mtDNA deletions but identified a patient with a slowly progressive myopathy harboring relatively high (32%) levels of the duplication in skeletal muscle, raising the possibility that the duplication could be pathogenic per se, if present at relatively high levels. Torroni et al. (1994) identified low levels of the same duplication in normal Caucasians harboring polycytosine stretches in the mtDNA 567–573 region that were longer than the standard "Cambridge" (Anderson et al. 1981) mtDNA haplotype (i.e., at least nine cytosines).

To address the potential pathogenic role of this small duplication in the mtDNA regulatory region, we created a cell line homoplasmic for the duplication. This model system allowed us to analyze several structural and functional properties of the human mtDNA promoter region.

Material and Methods

Cell Lines

The human osteosarcoma-derived cell line 143B(TK^-) and its mtDNA-less derivative 143B/206 (ρ°) were a kind gift from Dr. Michael P. King (Columbia University, New York). Growth conditions and the characterization of the 143B/206 ρ° cell line have been described elsewhere (King and Attardi 1989). Transmitochondrial

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cybrids were maintained in Dulbecco's modified Eagle medium (DMEM) and 4.5 g glucose/liter, supplemented with pyruvate but without uridine, except when functional or molecular studies were to be performed; in these latter cases, uridine (50 μg/ml) was added to the medium 48 h before the experiment, to avoid potential differences, in cellular growth or performance, due to its absence. A fibroblast line from a patient harboring an heteroplasmic (10%) mtDNA containing the 260dup has been obtained and characterized elsewhere (Manfredi et al. 1995). The selected clones used in the present study were frozen into several aliquots that were thawed to perform specific experiments (including DNA analysis). This procedure assured that no significant time in culture elapsed between the different analyses. Clone SUB62, homoplasmic for a pathogenic point mutation at mtDNA position 3256 within the $tRNA^{Leu(UUR)}$ gene, has been characterized elsewhere and was included in this study as a respiratory-deficient control (Hao and Moraes 1996).

Production and Genetic Characterization of Transmitochondrial Cybrids

Enucleated fibroblasts from the patient were fused to the mtDNA-less 143B/206 cell line as described elsewhere (King and Attardi 1989). Growth of parental cell lines was inhibited either by bromodeoxyuridine (fibroblasts and hybrids) or by the lack of uridine in the medium (143B/206 ρ°). Individual cybrid colonies first were analyzed by collecting a few (~100) cells from the initial foci, exposing their DNA by a micro–alkaline lysis procedure (Li et al. 1991), and genotyping the mtDNA for the 260dup as described elsewhere (Manfredi et al. 1995). Clones showing an amplified band corresponding to the duplication were analyzed by Southern blot and/or were recloned. Subsequent clones were genotyped by the same procedure.

Respiratory-Function Assays

Oxygen consumption was measured in a 5-ml reaction chamber (Yellow Springs Instruments) heated to 37°C and equipped with a Clark-type platinum polarographic electrode. Measurements were made with 5×10^6 exponentially growing cells (in the presence of uridine), resuspended in 2.5 ml of DMEM, without glucose, and 5% dialyzed FBS as described elsewhere (King and Attardi 1989). Respiratory-complex activity was measured in mitochondrial fractions isolated by standard methods (Fisher and Clayton 1985). Complexes I+III, II, II+III, and IV and citrate synthase were measured as described elsewhere (DiMauro et al. 1987).

Northern Blotting

Total RNA of each cybrid line was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi 1987). Twenty micrograms of total RNA were electrophoresed on 1.2% agarose/5% formaldehyde gels and were transferred to a nylon membrane. The filters were hybridized overnight at 42°C with specific mtDNA probes. For detection of 7S RNA, we used an oligonucleotide corresponding to mtDNA positions 242–280. For detection of an intermediate transcript originating from the heavy-strand (H-strand) promoter (HSP), we used an oligonucleotide corresponding to mtDNA positions 619–577 within the $tRNA^{Phe}$ gene. One hundred picomoles of each oligonucleotide were end-labeled with [γ-^{32}P]ATP and were added to the hybridization solution, at 5×10^5 cpm/ml. The probe for the detection of ND1 mRNA was obtained by gel purification of a 786-bp fragment (corresponding to mtDNA positions 3337–4121) produced by digestion of a 1,426-bp PCR fragment (primers spanning positions 3116–3125 and 4542–4521) by *Rsa*I and *Eco*RI. A 1.9-kb γ-actin mRNA probe was obtained by *Eco*RI digestion of a cloned insert (Erba et al. 1988). The purified fragment was ^{32}P-labeled by the random-primer method (Boehringer Mannheim). Northern hybridizations were performed in 50% formamide, 5 × Denhardt's solution, 0.1% SDS, 100 μg denatured salmon sperm DNA/ml, 25 mM sodium phosphate pH 6.8, and 5 × SSC and were washed twice in 1 × SSC and 0.1% SDS at room temperature for 1 h, then in 0.25 × SSC and 0.1% SDS at 55°C for 1 h, and finally in 0.1 × SSC at 55°C for 1 h. The northern blot subsequently was hybridized and stripped for a H-strand partially processed transcript, 7S RNA, ND1, and γ-actin probes.

Standard and Alkaline Southern Blot

To estimate the ratio of regulatory and nonregulatory mtDNA regions to nuclear DNA, ~5 μg of total DNA extracted from exponentially growing cells were digested with *Hin*dIII or *Eco*RI, were electrophoresed through a 0.8% agarose gel, were transferred to Zeta-Probe GT membranes (Bio-Rad), and hybridized simultaneously with three ^{32}P-labeled probes. The first probe was a 260-bp PCR fragment encompassing the duplicated mtDNA D-loop region (nucleotide positions 336–335); the second probe was a 1.9-kb PCR fragment corresponding to mtDNA positions 8289–10256); and the third probe was a 5.8-kb *Eco*RI insert from a construct containing the nuclear-encoded 18S rDNA gene (Wilson et al. 1978). The fragments were labeled with a random-primer labeling kit (Boehringer Mannheim). Filter hybridization was performed as recommended by the manufacturer (Bio-Rad), with 1×10^5 cpm of each mtDNA probe/ml and 5×10^5 cpm of the nuclear rDNA probe/ml (specific activities of ~$0.7–1 \times 10^8$ cpm/μg). In the *Hin*dIII digest (corresponding *Eco*RI digest sizes are given in parenthesis), the nuclear rDNA migrates as

a 13-kb (5.8-kb) band, the mtDNA promoter region as a 10.2-kb (8.0-kb) band, and the nonregulatory region, referred to as the "COX III region," as a 5.5-kb (7.4-kb) band. The ratio between the three bands was determined by digital scanning of a shortly exposed x-ray film and by quantitation with the software IMAGE 1.57 (National Institute of Mental Health shareware). The patient-type identity of mtDNA present in transmitochondrial cybrids was confirmed by RFLP analysis of a *Pvu*II polymorphism previously identified in this patient (Manfredi et al. 1995).

For the analysis of 7S DNA, 5 μg of total DNA from each cell line were electrophoresed through a 1.2% alkaline agarose gel (Sambrook et al. 1989) and were transferred to a nylon membrane. The filter was hybridized with a ^{32}P-labeled probe corresponding to mtDNA positions 15803–335.

DNA Sequencing

PCR fragments amplified with the primers corresponding to mtDNA positions 336–355 and 1071–1050 were used to amplify the promoter region. DNA fragments were purified from agarose gels by the QUIEX kit (Quiagen) and were sequenced directly in an ABI automatic sequencer using fluorescent dideoxynucleotides.

Immunoblotting

Immunoblottings were performed by use of an affinity-purified polyclonal antibody against mitochondrial transcrition factor A (mtTFA) (Parisi et al. 1993). Mitochondria were isolated from cultured cells as described elsewhere (Fisher and Clayton 1985). Different amounts (38.4, 25.6, 12.8, and 6.4 μg) of mitochondrial proteins were separated onto 12% SDS-PAGE gels and were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Prestained protein standards were used as molecular-weight markers and to provide visual confirmation of transfer efficiency. PVDF blot membranes were incubated for 1 h with 10% milk in PBS with 0.05% of Tween 20 as blocking agents. Membranes were then incubated with mtTFA polyclonal antibody for 14 h at 4°C and subsequently were incubated with anti-rabbit IgG conjugated to alkaline phosphatase (Sigma). Bands were developed by incubation with 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt and nitroblue tetrazolium chloride (GIBCO BRL) for 10 s.

Results

Isolation of Transmitochondrial Cybrid Line with the 260dup mtDNA

To better understand the effect of the mtDNA 260dup (fig. 1) in mitochondrial function, we tried to isolate a cybrid cell line containing exclusively mutated mtDNAs. Our starting material was a fibroblast line from a patient described elsewhere (Manfredi et al. 1995), containing only 5%–10% mutated mtDNA. After fusion of enucleated patient fibroblasts with the 143B/206 ρ° line, none of 60 independent cybrid clones showed the presence of the mtDNA duplication on Southern blot analysis. Using PCR, we identified clones with low levels of the duplication. These cybrid clones were submitted to two additional recloning steps. After the first round of recloning, individual clones showing higher levels of the duplication on PCR were selected for the second round. This approach led to the isolation of a clone with essentially only mutated mtDNA, as judged by overexposure of a Southern blot (clone CA17.1), and of a few others with lower percentages of mutated mtDNA (fig. 2 and table 1). The presence of a *Pvu*II polymorphism in the cybrid clones confirmed that the mtDNA originated from the patient's cells (not shown). We maintained and propagated a single 75-cm^2 flask of clone CA17.1 for a period of 2 mo, collecting, after every other passage, cells for DNA extraction (for a total of 26 passages). All DNA samples showed an identical mtDNA pattern on Southern blot, indicating that no new rearrangements (e.g., either loss of the duplication or large-scale deletions) occurred during this period (data not shown).

Oxidative-Phosphorylation Function in Clone CA17.1

We assessed the effect of the 260dup on mitochondrial function, by three independent approaches (fig. 3). The growth of clone CA17.1 was not affected by the absence of uridine, suggesting that the oxidative-phosphorylation system was at least partially functional (fig. 3*A*). Oxygen consumption, a sensitive index of respiratory-chain function, was unaltered in clone CA17.1 compared with controls and transmitochondrial cybrids containing exclusively wild-type mtDNA (fig. 3*B*). Enzyme activities of different respiratory complexes also were comparable to those of clones containing no or low levels of the 260dup mtDNA (fig. 3*C*).

mtDNA Polymorphisms Associated with the 260dup

Torroni et al. (1994) noticed that the 260dup is associated with the presence of a longer polycytosine stretch around mtDNA position 570. Sequence analysis showed that cells from our patient had an extremely long polycytosine stretch in this region. Moreover, we detected in muscle a mixture of mtDNA species containing different sizes (8–12 C's) of the polycytosine stretch, making the sequence analysis unreadable in PCR fragments spanning this region (fig. 4*B*). Fibroblasts, white blood cells from the patient, and clone CA17.17 (containing 29% 260dup mtDNA) showed an identical pattern (data not shown). A control containing only six C's did not show polyplasmy (fig. 4*A*). Clone CA17.1 was also polyplasmic in this region, but there was a clear (~80%) pre-

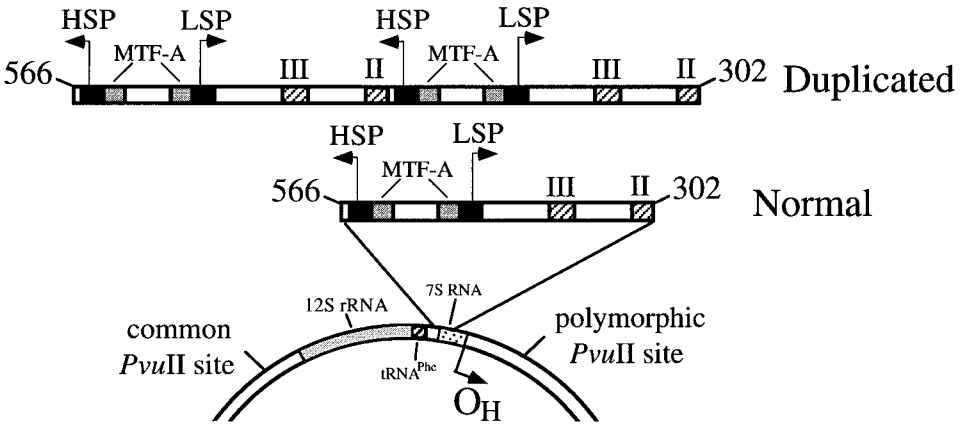

Figure 1 Location of the 260dup in the human mtDNA regulatory region. The diagrammed arch represents the circular 16,569-bp human mtDNA, showing the location of the 260dup. Blackened boxes on the duplicated region represent the mtDNA major HSPs and the L-strand major promoters (LSP); and hatched boxes represent binding sites for mtTFA, as well as the evolutionarily conserved sequence boxes III and II. The diagram also illustrates the locations of the origin of the H-strand replication (O_H), the "common" *Pvu*II site at nucleotide 2650, the polymorphic site at nucleotide 16239, and the genes for $tRNA^{Phe}$, 12S rRNA, and 7S RNA.

dominance of mtDNAs containing 11 C's. There also were less abundant mtDNA populations containing 10 C's and 12 C's (fig. 4*C*). The apparent polyplasmy was not an artifact of PCR amplification or sequencing (e.g., polymerase stuttering), because the sequence from a PCR fragment amplified from a cloned DNA segment containing 12 C's was homogeneous (data not shown). The second region (nucleotides 302–315) involved in the generation of the duplication junction also was longer, but homoplasmic, in our patient (eight C's, one T, and six C's), compared with either the published human mtDNA sequence (seven C's, one T, and five C's) or our control sequence (seven C's, one T, and six C's) (data not shown).

mtDNA Copy Number in Clone CA17.1

Because mtDNA replication depends on transcription in order to generate a replication primer (Clayton 1991), the duplicated region that includes the major promoters and binding sites for mtTFA is a candidate for the control of mtDNA copy number by the nucleus. We measured the relative mtDNA levels in clone CA17.1 and controls by probing a Southern blot simultaneously with three different probes corresponding to (1) the 260dup region, (2) a nonduplicated mtDNA region, and (3) a nuclear gene (see Material and Methods). The results indicate that the nonduplicated mtDNA region in clone CA17.1 is present at levels comparable to those in controls, whereas the promoter region:nuclear DNA ratio was increased (fig. 5).

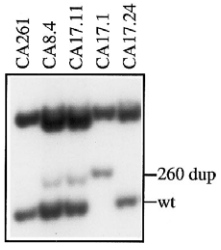

Figure 2 Isolation of transmitochondrial cybrids with the 260dup mtDNA. Shown is Southern analysis of *Rsa*I-digested DNA extracted from different transmitochondrial cybrid lines. Cell lines containing different percentages of the 260dup mtDNA were identified by use of a ^{32}P-labeled PCR fragment, corresponding to the duplicated D-loop and neighbor regions, as a probe (mtDNA positions 13956–175). Band numbers are indicated on the right.

Transcription and Promoter Selection in the 260dup mtDNA

Steady-state levels of the mtTFA can, in certain circumstances, be regulated by the levels of its binding sites in the mtDNA. Cells with low levels of mtDNA lack mtTFA (Larsson et al. 1994). Hence, mtTFA levels could be up-regulated if the number of binding sites in the mtDNA is doubled, such as in clone CA17.1. However, the steady-state levels of mtTFA, measured by western blot, were not different from those in control cell lines (fig. 6).

To analyze promoter usage for H-strand transcription, we probed a total RNA blot with an oligonucleotide corresponding to sequences complementary to the $tRNA^{Phe}$ gene. Although the tRNA run out of the gel shown in figure 7, this probe detected several large intermediate transcript species, the smallest with the approximate length of $tRNA^{Phe}$ + 12S rRNA + $tRNA^{Val}$, probably including the few nucleotides between the transcription start site and the $tRNA^{Phe}$ region (fig. 7*A*). Clone CA17.1 showed two forms of this intermediate

Table 1

Promoter Usage in Duplicated mtDNA Molecules

Cell Line	260dup mtDNA (%)	L-Strand RNA/γ-Actin	H-Strand RNA/γ-Actin (ND1/γ-Actin)	Usage of Distal Promoter in Transcription[a] (Predicted, if No Bias[b]) (%)	
				L-Strand	H-Strand
143B	Undetectable	.45	.41 (1.01)	...	...
CA261	Undetectable	.81	.46 (.53)	...	...
CA17.1	100	.63	.72 (.94)	36 (50)	45 (50)
CA8.4	11	1.02	.62 (.92)	2 (6)	5 (6)
CA17.17	29	.96	.79 (.90)	8 (15)	13 (15)
SUB62[c]	Undetectable	1.44	.31 (.53)	...	...

[a] The distal promoter is the promoter producing the larger transcript.
[b] Based on relative levels of the 260dup mtDNA, under the assumptions of equal usage of promoters and equal stability of transcripts.
[c] A respiratory deficient control homoplasmic for the pathogenic C3256T mtDNA mutation.

transcript (the second form being ~260 nucleotides longer). The band corresponding to the longer form was only 5% less intense than the smaller band, suggesting that the two H-strand major promoters present in the mutated molecule are similarly efficient in initiating transcription. We also did not detect a significant change in the steady-state levels of ND1 mRNA, another H-strand–coded gene (table 1).

Transcription from the light-strand (L-strand) promoter was assessed by measuring the levels of 7S RNA, a 210-nucleotide-long RNA that includes the transcription start site. Northern blot analysis (fig. 7*B*) showed an RNA species in clones CA17.1, CA8.4, and CA17.17 that was ~260 nucleotides longer than the normal 7S RNA. Both RNA species were present at similar levels, even though the signal originating from the distal promoter was ~30% weaker than the signal originating from the proximal promoter (fig. 7*B* and table 1). Overall levels of L-strand intermediate transcripts detected by this probe (i.e., originated from proximal plus distal promoters) were not significantly altered in clone CA17.1 (table 1).

L-Strand Transcripts as Primers for mtDNA Replication

An L-strand transcript normally is processed to form a replication primer (Chang and Clayton 1985). This 217-nucleotide RNA primer subsequently is extended by DNA polymerase that, in most cases, does not proceed to a complete replication cycle but, rather, aborts after ~600 nucleotides. Denaturing Southern blot showed that clone CA17.1 had normal sized "nascent D-loop DNA strands" at levels comparable to those of cell lines without the 260dup (fig. 8), suggesting that L-strand transcripts from both promoters can give rise to

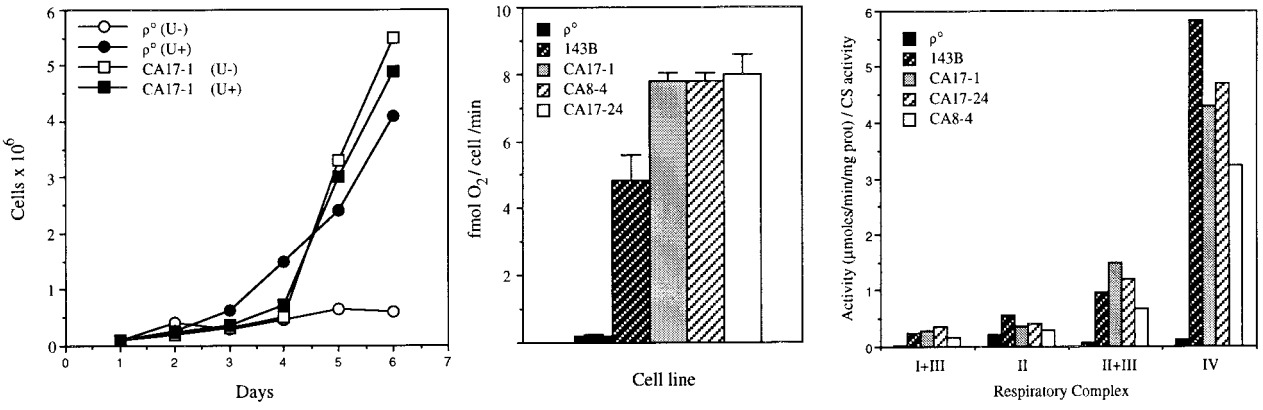

Figure 3 Mitochondrial functional assays in transmitochondrial cybrid clones. *A,* Growth curves of clones 143B/206 (ρ°) and CA17.1 in the presence (+U) and absence (−U) of uridine. *B,* Rates of oxygen consumption per cell, of 143B, 143B/206, and the indicated transmitochondrial cybrid lines, with error bars representing ±SD of three determinations. *C,* Spectrophotometrically determined activity of different respiratory complexes in isolated mitochondria normalized to the activity of the matrix enzyme citrate synthase.

a functional primer for replication. The 3′ end of the D-loop strand can have variable lengths (Chang and Clayton 1985), but only clone CA17.17 and the parental 143B lines showed a broadening of the band, suggesting the presence of different-sized species. We do not have an explanation for these differences.

Discussion

Effect of mtDNA Duplications on Mitochondrial Function

Tandem duplications of mtDNA regions have been associated repeatedly with a subgroup of mitochondrial disorders, which include Kearns-Sayre syndrome, Pearson syndrome, and myopathy with diabetes (Poulton and Holt 1994). However, there have been no reports directly linking mtDNA duplications to an oxidative-phosphorylation dysfunction. One of such mutations, a small duplication involving the promoter region, has been the object of much speculation. Brockington et al. (1993) have suggested that the presence of the 260dup would predispose mtDNA molecules to acquire large deletions. Lee at al. (1994) have suggested that small duplications in the mtDNA regulatory region are age related and associated with mtDNA deletions. Manfredi et al. (1995) have found no association between the mtDNA 260dup and mtDNA deletions but have suggested that, if present at relatively high levels, the 260dup could have a direct effect in mitochondrial function. Torroni et al. (1994) have detected low levels of the same mtDNA abnormality in a specific Caucasian haplotype, but, because of the low levels, they could not rule out a pathogenic role for the 260dup if it were present at higher levels.

We have generated a cell line (CA17.1) harboring essentially homoplasmic levels of the 260dup mtDNA. This cell line did not show a defect in oxidative phosphorylation, suggesting that the 260dup probably is not pathogenic. It is still possible that the 260dup may affect specific cell types (such as muscle or CNS) preferentially, similarly to the way in which some mtDNA mutations affecting the optic nerve have a very subtle effect in the respiration of cultured cells (Hofhaus et al. 1996). However, previously this discrepancy has not been observed with mtDNA mutations affecting muscle and causing RRF.

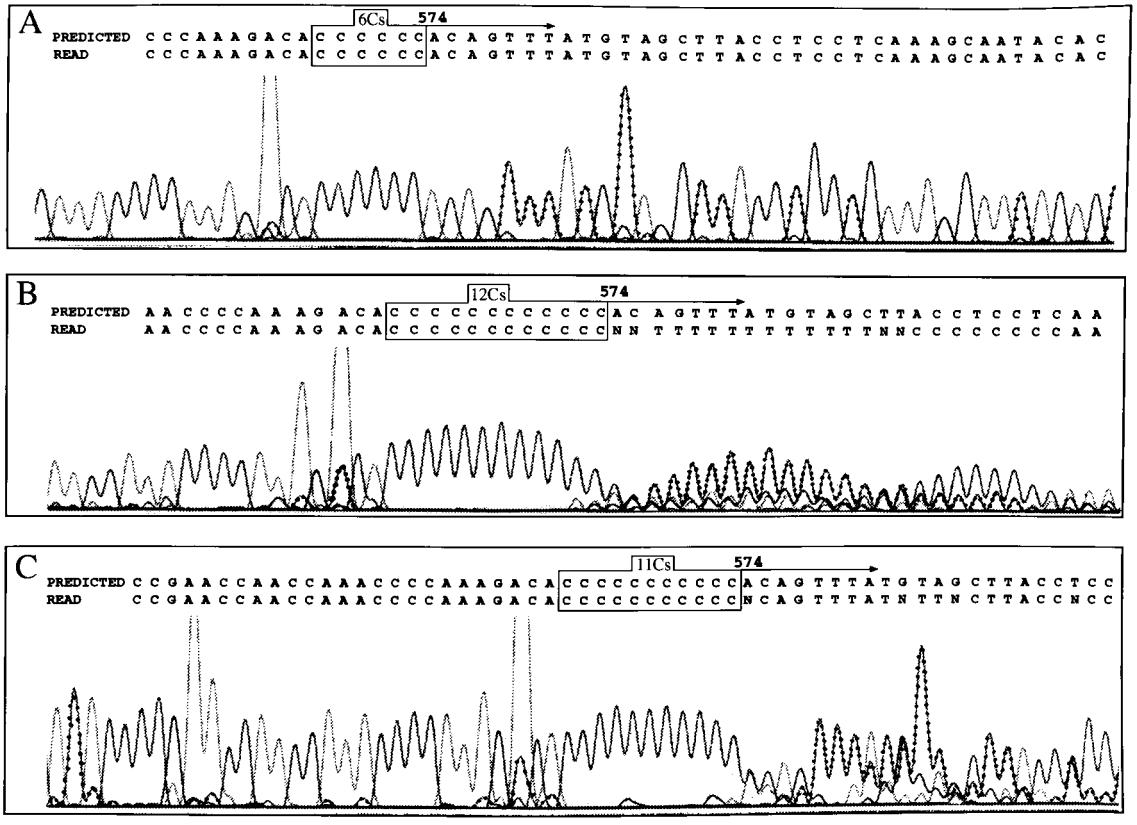

Figure 4 mtDNA polyplasmy of a cytosine stretch involved in the generation of the 260dup. An expanded and polyplasmic polymorphism was identified between mtDNA positions 569 and 574 in our patient's tissues. Shown is the DNA sequence obtained from a PCR-amplified fragment from an unrelated control (*A*), muscle from our patient (*B*), and clone CA17.1 (*C*). Note the unreadable nature of the sequence (because of polyplasmy) beyond the polycytosine stretch around position 570 in the patient's muscle.

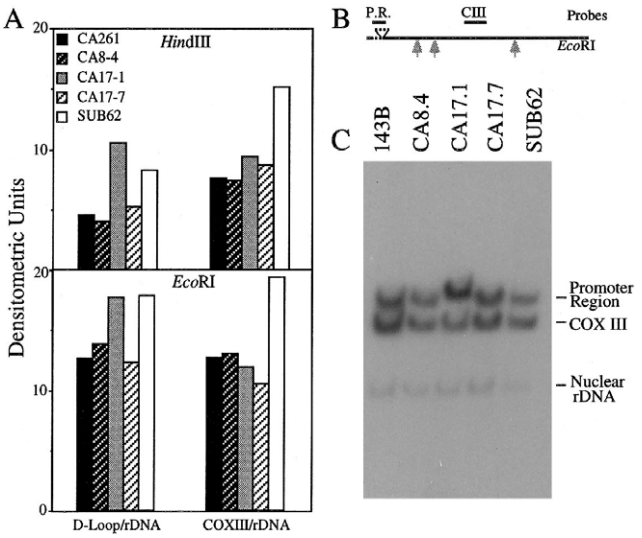

Figure 5 mtDNA levels associated with the 260dup. DNA samples extracted from exponentially growing cells were digested with *Hin*dIII or *Eco*RI, were electrophoresed through a 0.8% agarose gel, were transferred to nylon membranes, and hybridized simultaneously with three ^{32}P-labeled probes: one probe encompassing the duplicated mtDNA D-loop region, a second probe corresponding to a nonduplicated mtDNA COXIII gene region, and a third probe complementary to the nuclear-encoded 18S rDNA gene (see Material and Methods). *A,* Ratios of the duplicated and nonduplicated mtDNA regions to nuclear DNA for the *Eco*RI (panel *C*) and *Hin*dIII (not shown) digestions. *B,* Linearized representation of the circular mtDNA, showing the location of the 260dup, *Eco*RI sites (*arrows*), and the probes used in the experiment (P.R. [i.e., promoter region] and CIII [i.e., COX III]). *C,* Southern blot results obtained with *Eco*RI digestion. The origin of the bands (which is based on size) is indicated on the right. SUB62 is a respiratory-deficient cell line with a tRNA$^{Leu(UUR)}$-gene mutation (see Material and Methods).

Both Brockington et al. (1993) and our previous report (Manfredi et al. 1995) showed the presence of RRF in patients' muscle containing relatively higher levels of the 260dup mtDNA (5% 260dup mtDNA in Brockington et al.'s patient and 32% in ours). In view of the age of the two subjects (67 and 58 years old, respectively), these RRFs could have been associated with age-related mtDNA deletions (Cortopassi and Arnheim 1991; Johnston et al. 1995). This possibility also would explain the in situ hybridization experiment by Brockington et al., showing RRF underexpressing COX II and ND5, relative to ND6, in the patient with 5% 260dup mtDNA. Although those authors speculated that this pattern of expression could be a consequence of the 260dup on HSP function, our results showing normal H-strand transcription from the 260dup mtDNA favor the age-related mtDNA-deletion association.

Our present study raises further doubts about the potential etiologic role of mtDNA duplications. Although, in principle, duplications cause genes to fuse and thereby create dominant-acting hybrid-gene products (Poulton et al. 1993), "hybrid products" do not appear to behave in a dominant fashion in heteroplasmic mtDNA deletions (Sciacco et al. 1994). Our observations illustrate the need for in vivo studies to test such models. A similar 260-bp duplication in the mtDNA control region of the cyprinid fish, *Cyprinella spiloptera,* has been reported (Broughton and Dowling 1994). Individuals containing two or three copies of the region have been identified, but none contains only one copy. It is interesting to note that our patient also had a small mtDNA population harboring three copies of the promoter region in muscle (Manfredi et al. 1995). In addition, heteroplasmic repeated sequences in the mtDNA D-loop region have been reported in sturgeon (Burocker et al. 1990), rabbit (Mignotte et al. 1990), green monkey (Hayasaka et al. 1990), and pig (Madsen et al. 1993). These are not associated with mitochondrial dysfunction. It remains to be determined whether the prevalence of tandem duplications in the D-loop region has any biological significance.

Insights into mtDNA Promoter–Region Stability and Function

Torroni et al. (1994) identified, in a polycytosine stretch between positions 569–573, a length polymorphism associated with the presence of the 260dup mtDNA. Stretches containing more than seven C's were associated with the presence of PCR-detectable levels of the duplication. Our patient's tissues (muscle, fibroblasts, and white blood cells), as well as transmitochondrial cybrids, had a polyplasmic population of mtDNA molecules with very long stretches of C's (8–12) between positions 569 and 573. Clone CA17.1 was enriched in an mtDNA population harboring 11 C's, suggesting that a molecule(s) containing 11 C's underwent

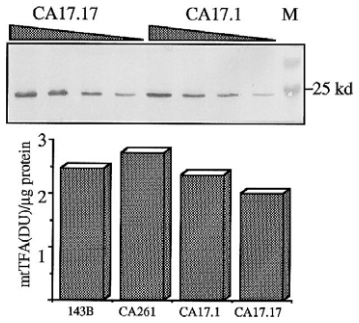

Figure 6 mtTFA levels in transmitochondrial cybrids containing the 260dup mtDNA. The steady-state levels of mtTFA in different cell lines was determined by western blot analysis. Serial twofold dilutions of purified mitochondria solubilized in SDS were subjected to PAGE and were electrotransferred to a PVDF membrane. The blot was incubated with an affinity-purified antibody against the human mtTFA, as described in Material and Methods. The lower panel shows the relative levels of mtTFA per microgam of mitochondrial protein.

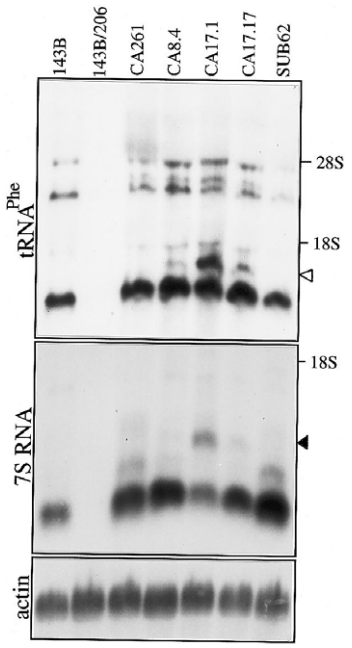

Figure 7 Transcriptional analysis of the duplicated promoter region. Autoradiograms of total RNA, extracted from the cell lines listed at the top of each lane, were electrophoresed through a 1% agarose gel and hybridized to different probes, shown on the left of each panel. In the upper panel, although the $tRNA^{Phe}$ run out of the agarose gel, the probe was able to detect partially processed transcripts, which include the H-strand transcription-initiation site, $tRNA^{Phe}$, and downstream sequences. The most prevalent RNA species had the lowest molecular weight, with a size compatible with a transcript extending from the HSP to the $tRNA^{Val}$. The unblackened arrowhead indicates a transcript ~260 nucleotides longer than the latter, and it was observed only in clones with the 260dup. In the middle panel, the blackened arrowhead indicates a transcript ~260 nucleotides longer than the 7S RNA observed only in clones with the 260dup. In the lower panel is nuclear-coded γ-actin. The ethidium bromide–determined positions of 28S and 18S rRNAs are shown on the right of two panels. Band assignments were based on molecular weight and probe specificity (see Material and Methods).

the original duplicative event. However, this same cell line also had low levels (~20%) of mtDNAs harboring 10 or 12 C's. It is possible that the duplication occurred independently in molecules containing different numbers of C's, but it also possible that the same 11 C's–containing mtDNA returned to a metastable level of polyplasmy. Torroni et al. (1994) found two different sizes of this polycytosine stretch in two different tissues from the same individual. However, they reported the sequences as homoplasmic within the same tissue. Our results show that individuals with mtDNA containing long polycytosine stretches between positions 569 and 573 can be polyplasmic. We did not detect a previously reported polyplasmy between positions 303 and 315 (Hauswirth and Clayton 1985) in our patient's tissues. It is interesting to note that the DNA sequence at the breakpoint of the 260dup is identical to the homoplasmic repeat between mtDNA positions 303 and 315 (i.e., eight C's, one T, and six C's). This is similar to the breakpoint described by Brockington et al. (1993) but is different from the breakpoint described by Torroni et al. (1994), where the junction was similar to that in region 569–573. In either case, slip misparing is the most likely mechanism for the generation of the 260dup. The duplication itself seems to be relatively stable in culture, since clone CA17.1 did not show any detectable reversion to a single-copy promoter region during 2 mo of continuous growth.

Although the 260dup mtDNA did not impair mitochondrial function significantly, it provided a unique model system to study the human mtDNA promoter region in vivo. Steady-state levels of H-strand transcripts were not altered by the presence of two major promoters, and both were similarly efficient in promoting transcription initiation. Steady-state levels of downstream H-strand transcripts also were not affected. Overall, steady-state levels of L-strand transcripts also were not elevated. The RNA made from the distal promoter was slightly reduced when compared with that made from the proximal promoter, which may reflect the influence of neighboring regions on DNA topology, binding of trans-acting factors such as mtTFA or the RNA polymerase, or RNA stability.

The essentially identical levels of transcripts from the two strand-specific promoters, as well as the unaltered overall levels of transcripts in a cell line with twice the number of promoters, could have different explanations. However, limiting levels of RNA polymerase is a likely mechanism to explain our results, since the enzyme ran-

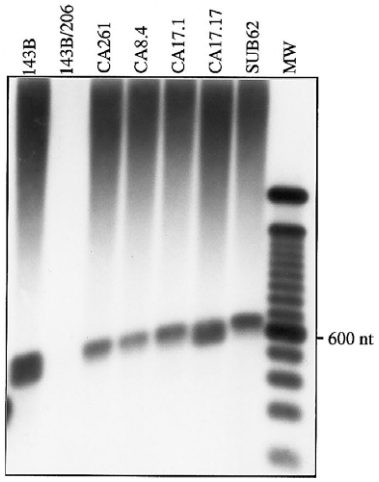

Figure 8 Replication initiation of mtDNA in transmitochondrial cybrids harboring the 260dup. DNA extracted from different cell lines was denatured and subjected to an alkaline Southern blot analysis (see Material and Methods). Shown is an autoradiogram after hybridization with a D-loop–specific probe. Markers correspond to a 100-nucleotide ladder.

domly would initiate transcription from one promoter, with a low probability that another RNA polymerase would be engaged in transcription initiation in the same mtDNA molecule.

mtDNA and the nascent D-loop DNA-strand levels also were unaltered in clone CA17.1, probably because of the "normal" levels of RNA primers generated by the combined L-strand transcription from both promoters. These results suggest that the activity of the mtRNA processing enzyme (Clayton 1991) is independent of the original transcript size. The mechanisms controlling mtDNA copy number are unknown, and the results presented here do not support the concept that the nucleus is "sensing" the levels of the mtDNA control region, since mtDNA levels were unaltered by the presence of the promoter-region duplication.

Although we could not find any deleterious effect for the 260dup, it is curious that this mtDNA polymorphism never has reached homoplasmy in humans, even though it is formed repeatedly (Torroni et al. 1994). This observation may suggest that the 260dup has some disadvantage in a complete human organism, either at the molecular or, less likely, at the functional level (e.g., in a tissue-specific manner). Naturally occurring mtDNA mutations, such as the one described in this report, have shed—and will continue to shed—light on the potential mechanisms underlying mitochondrial disorders and will provide unique models for the in vivo study of mitochondrial biogenesis in mammals.

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