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Rearrangements of Human Mitochondrial DNA (mtDNA): New Insights into the Regulation of mtDNA Copy Number and Gene Expression

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Mitochondria from patients with Kearns–Sayre syndrome harboring large-scale rearrangements of human mitochondrial DNA (mtDNA; both partial deletions and a partial duplication) were introduced into human cells lacking endogenous mtDNA. Cytoplasmic hybrids containing 100% wild-type mtDNA, 100% mtDNA with partial duplications, and 100% mtDNA with partial deletions were isolated and characterized. The cell lines with 100% deleted mtDNAs exhibited a complete impairment of respiratory chain function and oxidative phosphorylation. In contrast, there were no detectable respiratory chain or protein synthesis defects in the cell lines with 100% duplicated mtDNAs. Unexpectedly, the mass of mtDNA was identical in all cell lines, despite the fact that different lines contained mtDNAs of vastly different sizes and with different numbers of replication origins, suggesting that mtDNA copy number may be regulated by tightly controlled mitochondrial dNTP pools. In addition, quantitation of mtDNA-encoded RNAs and polypeptides in these lines provided evidence that mtDNA gene copy number affects gene expression, which, in turn, is regulated at both the post-transcriptional and translational levels.

INTRODUCTION

Large-scale human rearrangements of mitochondrial DNA (mtDNA; i.e., kilobase pair-sized partial deletions and duplications) are associated with a number of sporadic, mendelian, and maternally inherited human disorders (Schon *et al.*, 1997), the most prominent of which is Kearns–Sayre syndrome (KSS). KSS is a sporadic and ultimately fatal multisystem disorder characterized by progressive external ophthalmoplegia, pigmentary retinal degeneration, onset before age 20 y, ataxia, heart block, elevated cerebrospinal fluid protein, and diabetes mellitus (Kearns and Sayre, 1958; Berenberg *et al.*, 1977). Partial deletions of mtDNA in these patients were first described in 1988 (Holt *et al.*, 1988; Lestienne and Ponsot, 1988; Zeviani *et al.*, 1988), followed soon thereafter by the identification of partial duplications of mtDNA (Poulton *et al.*, 1989). It was determined later that deleted and duplicated mtDNAs coexist in a subset of KSS patients (Poulton *et al.*, 1993, 1995; Brockington *et al.*, 1995). Besides KSS, clinical phenotypes associated with large-scale mtDNA rearrangements include renal tubulopathy, cerebellar ataxia, and diabetes mellitus (Rötig *et al.*, 1992); progressive external ophthalmoplegia, myopathy, and diabetes (Dunbar *et al.*, 1993); diabetes and deafness (Ballinger *et al.*, 1992, 1994); and late-onset myopathy (Manfredi *et al.*, 1997). A partial triplication coexisting with a duplication has also been identified (Tengan and Moraes, 1998).

In any one patient harboring mitochondrial genomes with deletions and duplications, the two rearranged species are topologically related: the duplicated mtDNA is composed of two mtDNA molecules, a wild-type mtDNA and a deleted mtDNA, arranged head to tail (see example in Figure 1A). The only novel sequence in the duplicated mtDNA compared with wild-type mtDNA is at the boundary of the duplicated region, which is the same as the boundary present in the corresponding deleted mtDNA (Figure 1A, dashed lines), suggesting that the two molecules are generated through a common mechanism or that one may be derived from the other.

High levels of such giant deletions (which invariably remove at least one tRNA gene) are pathogenic. This conclusion is based on studies conducted both in vivo (Mita *et al.*,

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1989; Nakase *et al.*, 1990; Shoubridge *et al.*, 1990; Moraes *et al.*, 1991, 1992; Sciacco *et al.*, 1994) and in vitro (Moraes *et al.*, 1989b; Hayashi *et al.*, 1991; Sancho *et al.*, 1992). It is thought that deleted mtDNAs alter mitochondrial respiratory chain function for one of two reasons. First, if the novel fusion gene located across the deletion breakpoint is expressed (Hayashi *et al.*, 1991), it could potentially disrupt mitochondrial function, even if it were present at low levels (i.e., the novel gene product may act dominantly). Second, if the majority of mtDNAs in a cell or in an organelle are deleted, none of the polypeptide-coding mRNAs would be translated, because of an insufficient amount (or even total absence) of one or more required tRNA genes located within the deleted region (Nakase *et al.*, 1990; Hayashi *et al.*, 1991).

The mechanism of pathogenesis of duplicated mtDNAs, however, is less clear, because at least one copy of each mitochondrial gene is present in these molecules. Besides the potentially deleterious effects of an expressible fusion gene, changes in gene dosage could also render a duplicated mtDNA pathogenic. For example, transcription of the duplicated region could result in an excess or an imbalance in the levels of rRNAs, mRNAs, or tRNAs (King and Attardi, 1993), which might result in alterations in translation.

The analysis of transmitochondrial cytoplasmic hybrids (cybrids) containing mtDNAs with a partial duplication did not reveal that duplicated mtDNAs affected mitochondrial function adversely (Holt *et al.*, 1997). These investigators also found that the nuclear background of the cells could influence temporal shifts in heteroplasmy and that, at least in vitro, duplicated mtDNAs can give rise to triplicated mtDNAs.

To further investigate the pathogenesis and genetic consequences of mtDNA rearrangements, we have generated and analyzed transmitochondrial cells containing rearranged mtDNAs from KSS patients harboring wild-type, deleted, and duplicated mtDNAs propagated in the same parental nuclear background. Furthermore, the mtDNAs used in our study were isogenic, differing only by the rearrangements of the genome. Therefore, the influence of mtDNA sequence polymorphisms on the maintenance and expression of the mitochondrial genome was controlled in these studies.

MATERIALS AND METHODS

Throughout this paper, the term *genome* refers to a monomeric circle of mtDNA, unless otherwise indicated. The term *deleted region* refers to the segment of mtDNA that has been removed from wild-type mtDNA (Figure 1A, protruding "pie section"), and the resulting genome is termed a *deleted mtDNA*. The term *duplicated region* refers to the segment of mtDNA that has been inserted into wild-type mtDNA and that corresponds to the deleted mtDNA; the resulting genome is termed a *duplicated mtDNA*. The terms *wild-type cell line*, *duplication cell line*, and *deletion cell line* refer to transmitochondrial cells containing homoplasmic levels of the respective mtDNAs.

Patients

KSS patient 1 has been described previously (Wilichowski *et al.*, 1997, their patient 4). She harbored both deleted and duplicated mtDNAs in all tissues studied (Wilichowski *et al.*, 1997). The deletion was 7813 bp in size, located between nucleotide (nt) 7883 in the COX II gene and nt 15,696 in the Cyt *b* gene (notation of Anderson *et al.*, 1981; Figure 1A). KSS patient 2 has been described elsewhere (Moraes *et al.*, 1989a; Nakase *et al.*, 1990, their patient 2). Her tissues contained deleted mtDNAs, but no analyses were performed to determine whether duplicated mtDNAs were also present (Nakase *et al.*, 1990). The deletion was 7521 bp in size, located between nt 7983 in the COX II gene and nt 15,504 in the Cyt *b* gene.

Cell Culture

The 143B (ρ^+) and 143B206 (ρ^0) cells have been described previously (King and Attardi, 1989). Mitochondria from patient fibroblasts (see Table 1) were transferred to 143B206 cells as described (King and Attardi, 1989; King *et al.*, 1992). Cells were replated 24 h after fusion in Dulbecco's modified Eagle's medium containing 4.5 mg glucose/ml (DMEM; Life Technologies, Gaithersburg, MD) supplemented with 100 μg of 5-bromodeoxyuridine/ml and 5% dialyzed fetal bovine serum (FBS) (selective medium). The medium was changed to DMEM supplemented with 50 μg of uridine/ml, 100 μg of 5-bromodeoxyuridine/ml, and 5% FBS (nonselective medium) 9–11 d after fusion. Unless noted otherwise, cell lines were grown in DMEM supplemented with 50 μg of uridine/ml and 5% FBS.

To obtain cells homoplasmic for wild-type and rearranged species of mtDNA, heteroplasmic cybrid cells were treated with ethidium bromide (an inhibitor of mtDNA replication) as described (King, 1996). For patient 1, cells from a clonal transmitochondrial cell line (KAF4) containing wild-type, deleted, and duplicated mtDNA species were grown for 12 d in DMEM supplemented with 5% FBS, 50 ng of ethidium bromide/ml, and 50 μg of uridine/ml. This treatment results in a reduction of the mtDNA content to an average of less than one mtDNA molecule per cell (King, 1996). After ethidium bromide treatment, the cells were grown for 3 d in DMEM supplemented with 5% FBS and 50 μg of uridine/ml to permit the residual mtDNA to repopulate the cells. The cells were subsequently replated at low density (100 cells per 100-mm plate) in the same medium to facilitate the selection of individual cell colonies with glass cloning cylinders. Selected colonies were expanded for genetic analyses. The isolation of cell lines homoplasmic for rearranged mtDNA species from patient 2 has been described previously (King, 1996). The cell lines used in this study are listed in Table 1.

Growth curves of the cell lines were determined by seeding multiple 100-mm plastic Petri dishes with a constant number of cells in 10 ml of DMEM containing 4.5 mg of glucose/ml plus 5% dialyzed FBS or in the same medium supplemented further with 50 μg of uridine/ml and 110 μg of pyruvate/ml. Cells were also grown in DMEM without glucose but containing 0.9 mg of galactose/ml and 5% dialyzed FBS and also supplemented with 50 μg of uridine/ml alone, 110 μg of pyruvate/ml alone, both 50 μg of uridine/ml and 110 μg of pyruvate/ml, or neither uridine nor pyruvate. At various time intervals, cells from individual plates were trypsinized and counted.

DNA Analyses

Total DNA was extracted from exponentially growing cells as described (Davis *et al.*, 1986). For patient 1, rearranged mtDNA species were initially detected in cells by PCR amplification across the breakpoint region, using primers (5′→3′) corresponding to nt 7407–7429 (forward) and nt 15,885–15,860 (reverse) for 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. For Southern blot analyses, 2 μg of total DNA from both patients were digested with the restriction enzymes *Pvu*II or *Bam*HI (Boehringer Mannheim, Indianapolis, IN). Gel electrophoresis and capillary transfer (Zeviani *et al.*, 1988) to Zeta Probe membranes (Bio-Rad, Hercules CA) were performed as recommended by the manufacturer. The blots were hybridized with two human mtDNA-specific probes, generated by PCR and labeled with [α-^{32}P]dATP using a random-priming kit (Boehringer Mannheim): probe 1 (988 bp, from nt 1460–2447) and probe 2 (1060 bp, from nt 8239–9298). Hybridizing fragments were quantitated using a Bio-Rad GS-363 molecular imager system. Partial digestions of total DNA with *Pvu*II were performed using

serial dilutions of enzyme incubated for 1 h at 37°C and then subjected to Southern blot analysis as above. For DNA sequencing, PCR products were gel purified with a Wizard PCR-prep DNA purification system (Promega, Madison, WI), and dideoxy sequencing was performed using the *fmol* DNA cycle sequencing system (Promega), as suggested by the manufacturer.

Biochemical Analyses

Oxygen consumption by intact cells was determined as described (King *et al.*, 1992). ATP synthesis from isolated mitochondria was measured as described (Vazquez-Memije et al., 1996), using succinate as a substrate.

Determination of mtDNA Copy Number

Twenty independent cell samples from each cell line of patient 1 and 10 independent cell samples from each cell line of patient 2 were collected from exponentially growing cells at different times of passage. Cell suspensions containing 5×10^4 cells were brought to 1 ml with 50 mM Tris·HCl, pH 8 (25°C), 100 mM EDTA, and 100 mM NaCl, digested overnight at 50°C with 250 μg of proteinase K, and incubated with 0.5 M NaOH at 37°C for 16 h to hydrolyze RNA. Aliquots of 50 μl were spotted onto a Zeta-Probe GT membrane (Bio-Rad) for dot blot analysis, as recommended by the manufacturer. A standard curve was constructed with known amounts of closed circular HeLa cell mtDNA. The blots were hybridized sequentially with probes 2 and 1, as described above. Comparison of hybridization signals from the samples with those of the HeLa cell mtDNA standards was performed to determine mtDNA copy number and, hence, mass.

Analyses of Mitochondrial Translation Products

Exponentially growing transmitochondrial lines and 143B206 cells were labeled with Express^{35}S^{35}S protein labeling mix (>1000 Ci/mmol; New England Nuclear, Boston, MA) for 20–30 min in 4 ml of methionine- and cysteine-free DMEM supplemented with 100 μg of emetine/ml and 5% dialyzed FBS, as described (Chomyn *et al.*, 1991). The translation products were analyzed by electrophoresis of equal amounts of total protein (Lowry *et al.*, 1951) from each cell sample through 10% Tricine SDS-PAGE gels (Schägger and von Jagow, 1987), and subjected to fluorography on Eastman Kodak (Rochester, NY) XAR film. Quantitation of mtDNA-encoded polypeptides was performed with a GS-363 molecular imager system.

RNA Analyses

Total RNA was isolated from exponentially growing cells (Ullrich *et al.*, 1977) from all eight cell lines from patient 1 (Table 1) and was electrophoresed through three 1.2% agarose-formaldehyde gels, transferred to three Zeta-Probe membranes (Bio-Rad), and hybridized according to the manufacturer's suggested protocols. Each blot was hybridized first with a cytoplasmic β-actin cDNA (insert of pHFβA-1; Gunning *et al.*, 1983) labeled with [α-^{32}P]dATP, to correct for the amount of RNA loaded and was then hybridized sequentially with one of the three following sets of mitochondrial probes, labeled in the same manner, after removing each previously used probe: 1) ND1 (PCR fragment from nt 3494–4198), ND2 (nt 4876–5400), ND3 (nt 10,062–10,388), ND4 (nt 10,762–12,092), and ND5 (nt 12,340–13,341); 2) COX I (nt 6190–7267), COX II-out (outside the deleted region; nt 7586–7882), COX II-in (inside the deleted region; nt 7883–8262), COX III (nt 9744–9950), and Cyt *b*-out (outside the deleted region; nt 15,698–15,887); and 3) 12S rRNA (nt 658-1497), 16S rRNA (nt 1732–3140), and ATP 6 (nt 8657–9028). RNA from the eight cell lines was also electrophoresed through a 2.5% agarose-formaldehyde gel and blotted to a fourth membrane, which was probed first with the nuclear-encoded human cytoplasmic tRNA^{Met} gene (Santos and Zasloff, 1981) to correct for the amount of RNA loaded and then sequentially with probes (labeled by the hot-cycle PCR method; Mertz and Rashtchian, 1994) for mitochondrial tRNA^{Lys} (nt 8295–8364), tRNA^{Glu} (nt 14,674–14,742), tRNA^{Phe} (nt 531–618), and tRNA^{Ser} (nt 7445–7516).

Table 1. Cells and cell lines used in this study

Cell or cell line	Patient	mtDNA (%)		
		WT	Del	Dup
143B (ρ^+)	–	100	0	0
143B206 (ρ^0)	–	0	0	0
Fibroblasts	1	70	20	10
KAF4EB12.22	1	100	0	0
KAF4EB12.26	1	100	0	0
KAF4EB12.39	1	100	0	0
KAF4EB12.17	1	0	100	0
KAF4EB12.49	1	0	100	0
KAF4EB12.28	1	0	0	100
KAF4EB12.32	1	0	0	100
KAF4EB12.42	1	0	0	100
206/F4 3.13.44	2	100	0	0
206/F4 3.13.41	2	0	100	0
206/F4 3.13.46	2	0	100	0

RESULTS

Generation and Characterization of Cybrids

Cytoplasts derived from patient 1 fibroblasts containing 10% duplicated mtDNA, 20% deleted mtDNA, and 70% wild-type mtDNA were fused with human 143B206 cells lacking endogenous mtDNA (ρ^0 cells). Several hundred presumptive cybrid clones were obtained. After replating at low density in selective medium, 76 cell colonies were expanded and grown for genetic analysis, of which 27 contained rearranged mtDNAs, as determined by PCR analysis.

The wild-type, duplicated, and deleted mtDNAs in the 27 heteroplasmic clones from patient 1 were identified, and relative proportions of each species were quantitated by Southern blot analysis, using total DNA digested with either *Pvu*II or *Bam*HI and using probes corresponding to regions located either outside (probe 1) or inside (probe 2) the deleted region. This scheme allowed us to distinguish (and quantitate) unambiguously all three mtDNA species, even when they were present in a heteroplasmic mixture (Poulton *et al.*, 1993; Fromenty *et al.*, 1997). Among the 27 transmitochondrial clones containing rearranged mtDNAs, 19 clones contained both wild-type and deleted mtDNAs (ranging from 30 to 50% deleted mtDNA), 7 clones contained both wild-type and duplicated mtDNAs (ranging from 10 to 30% duplicated mtDNA), and 1 clone (KAF4) contained all three species (~40% duplicated, 60% deleted, and a trace amount of wild-type mtDNA).

To obtain cell lines homoplasmic for each of the three mtDNA species, KAF4 cells were treated with ethidium bromide for 12 d (King, 1996). Total cellular DNA from 35 cell lines obtained after ethidium bromide treatment and subcloning (denoted KAF4EB12 lines) was subjected to DNA analysis. Three cell lines contained 100% duplicated mtDNA, two contained 100% deleted mtDNA, and three

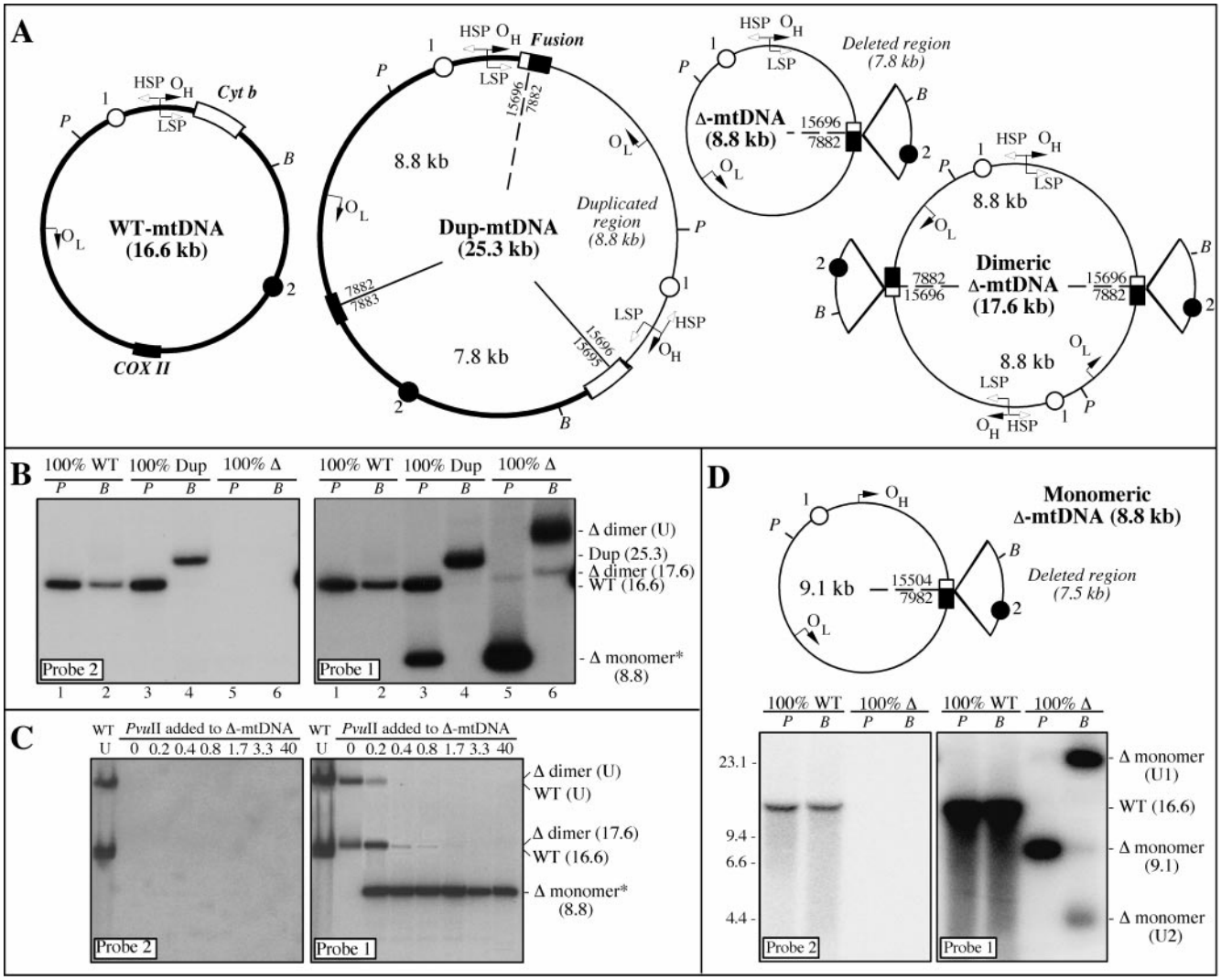

Figure 1. Rearranged mtDNAs. (A) Maps of the mtDNA species observed in cybrids from patient 1. The duplicated mtDNA (Dup-mtDNA) is composed of a full-length wild-type mtDNA (WT-mtDNA; thick arc) into which a subgenomic segment of wild-type mtDNA (i.e., the duplicated region) has been inserted (thin arc). On the deleted mtDNA (Δ-mtDNA, which is identical to the duplicated region), the protruding pie section denotes the deleted region. Only the COX II (solid box) and Cyt *b* (open box) genes involved in the rearrangement are shown (the breakpoint is indicated by a dashed line). Nucleotide positions associated with the rearrangement straddle the pie slices. Also shown are the origins of replication (O_H and O_L; solid arrows), the promoters of transcription (HSP and LSP; open arrows), the locations of the *Bam*HI (*B*) and *Pvu*II (*P*) restriction sites, and probes 1 (open circle) and 2 (solid circle) used in the Southern blot analyses. (B) Representative autoradiograms of Southern blot analyses of transmitochondrial cell lines from patient 1, containing 100% wild-type (WT; clone KAF4EB12.22), duplicated (Dup; KAF4EB12.28), and deleted (Δ; KAF4EB12.49) mtDNAs after digestion with *Pvu*II (*P*) or *Bam*HI (*B*) and hybridization with probe 1 or 2. The identity of each hybridizing fragment and its size (in kilobases) is indicated at right. The 8.8-kb band represents either linearized full-length Δ-mtDNA (in deleted lines) or the duplicated region in dup-mtDNA (in duplicated lines). U, uncut mtDNA. Lanes are numbered at the bottom. (C) Identification of dimeric species in deletion lines. Total DNA from deletion line KAF4EB12.49 from patient 1 was digested with successively smaller quantities of *Pvu*II (units indicated above each lane) before Southern blot analysis. Other notations are as in B. (D) Representative Southern blot analysis of wild-type (206/F4 3.13.44) and deleted (206/F4 3.13.41) cybrid lines from patient 2 (map of the deleted mtDNA above autoradiograms). U1 and U2, two different topological conformations of the uncut deleted monomeric mtDNA that migrated at two different positions in the gel. Other notations are as in B. Marker (λ DNA digested with *Hin*dIII) sizes, in kilobases, are at left.

contained 100% wild-type mtDNA (Table 1). The location of the rearrangement breakpoint in each cell line was confirmed by sequencing of appropriate PCR products. Of the remaining 27 cell lines, 20 were devoid of mtDNA and 7 were heteroplasmic.

A Southern blot analysis of the mtDNA of representative wild-type, deletion, and duplication cell lines is shown in Figure 1B. The cell line homoplasmic for wild-type mtDNA exhibited a single band of 16.6 kb after digestion with either *Pvu*II (Figure 1B, lane 1) or *Bam*HI

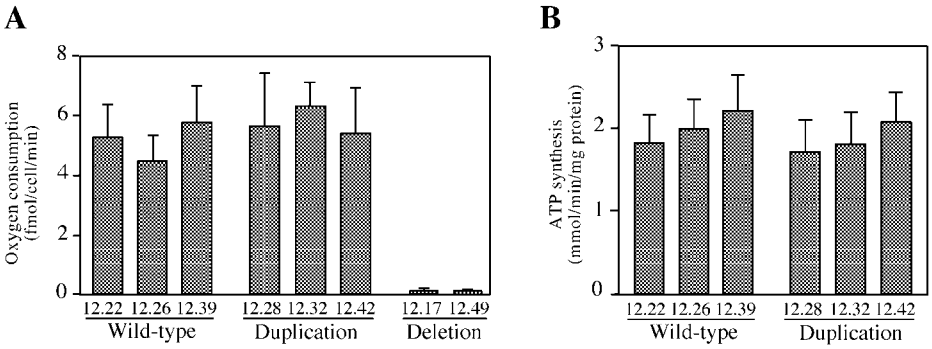

Figure 2. Biochemical function in homoplasmic cybrids from patient 1. (A) Rates of oxygen consumption per cell of the indicated cybrid cell lines (the digits under each bar denote the specific KAF4EB12.xx line analyzed; see Table 1) are shown (average of six measurements). (B) Rates of ATP synthesis of the indicated transmitochondrial cell lines are shown (average of four measurements per line). Each error bar represents 1 SD.

(Figure 1B, lane 2) and hybridization with either probe 1 or probe 2.

Digestion of total DNA from the three duplication cell lines with *Pvu*II and hybridization with probe 2 revealed a single band of 16.6 kb (Figure 1B, lane 3). With probe 1, however, in addition to the 16.6-kb band, a band of 8.8 kb was also observed (Figure 1B, lane 3). The *Bam*HI restriction products revealed a single band of 25.3 kb with both probes (Figure 1B, lane 4). These results indicated that this cell line was homoplasmic for the duplicated mtDNA.

Digestion of total DNA from the two deletion cell lines with either *Pvu*II or *Bam*HI revealed no restriction fragments hybridizing to probe 2 (Figure 1B, lanes 5 and 6), indicating that the mtDNA consisted solely of deleted molecules (this was also confirmed by PCR analysis; our unpublished results). When the *Pvu*II restriction products were hybridized with probe 1, a strongly hybridizing signal was observed migrating at 8.8 kb, with a trace of signal migrating between 16 and 20 kb (Figure 1B, lane 5). Digestion with *Bam*HI and hybridization with probe 1 yielded a strong signal migrating at approximately >30 kb and a weaker signal migrating at 16–20 kb (Figure 1B, lane 6), implying that these two hybridizing bands represented at least two forms of uncut deleted mtDNA. The >30-kb band migrated more slowly than expected for an uncut 8.8-kb circular molecule. To investigate the origin of this band, we performed *Pvu*II partial digestions of total DNA isolated from the two deletion cell lines. Southern blot analysis (Figure 1C) revealed that as the amount of *Pvu*II increased, the >30-kb band decreased. As the >30-kb band diminished, the band migrating at 16–20 kb first increased in abundance and then gradually dimished, whereas at the same time the 8.8-kb band increased; no other mtDNA species were detected. These results suggested that the band migrating at 16–20 kb is a linearized form of a dimer of deleted mtDNA (i.e., 17.6 kb in size; see Figure 1A), and that the band migrating at >30 kb is the uncut circular form of this dimer. Thus, both deletion lines contained exclusively deleted mtDNA, present predominantly, or even exclusively, in the dimeric form.

Transmitochondrial cell lines from patient 2 were treated for 13 d with ethidium bromide (denoted 206/F4 3.13 clones; King, 1996) to generate lines homoplasmic for wild-type and deleted mtDNAs. Southern blot analysis of total DNA isolated from these lines revealed that the mtDNA in the 100% deletion cell lines were present only as monomers (Figure 1D).

Biochemical Analyses

The respiratory capacity of the transmitochondrial cell lines was examined by determining the rate of oxygen consumption of intact cells (Figure 2A). The average rate of oxygen consumption of the lines containing 100% duplicated mtDNA (6.0 ± 0.8 fmol/cell per min) was similar both to that of the wild-type lines (5.2 ± 1.1 fmol/cell per min) and to 143B cells, the ρ^+ parent of the ρ^0 cell line (4.4 ± 0.6 fmol/cell per min; our unpublished results). However, the average rate of oxygen consumption of cybrid cell lines containing 100% deleted mtDNA (0.15 ± 0.05 fmol/cell per min) was significantly lower than that of the wild-type lines and was comparable with that in 143B206 ρ^0 cells (0.20 ± 0.03 fmol/cell per min; our unpublished results). Measurements of the rates of ATP synthesis in isolated mitochondria (Figure 2B) confirmed that the respiratory chain function of the duplication cell lines (1.9 ± 0.4 mmol of ATP/min per mg of protein) was not significantly different from that of the wild-type lines (2.0 ± 0.4 mmol of ATP/min per mg protein; $p > 0.05$ by Student's *t* test).

Growth Characteristics

Transmitochondrial cell lines homoplasmic for wild-type, duplicated, or deleted mtDNAs were analyzed for their growth properties, using culture conditions known to distinguish wild-type from respiratory-deficient cells, which require uridine (Desjardins *et al.*, 1986), pyruvate (King and Attardi, 1989), and glucose (Robinson, 1996) for normal growth. (ATP is generated largely by glycolysis when glucose is the substrate but by oxidative phsophorylation from mitochondria when galactose is the substrate.) Cell lines were examined for their growth characteristics in the presence or absence of some or all of these supplements. A representative analysis is shown in Figure 3. The three cell lines containing 100% wild-type mtDNA had similar growth characteristics, including population-doubling times, in all media tested (Figure 3, A and B). All cell lines containing 100% duplicated mtDNA had growth characteristics similar to wild-type lines in all media tested (Figure 3, C and D). The transmitochondrial lines containing 100% deleted mtDNA had a slightly reduced doubling time in complete medium when compared with wild-type and duplication cell lines and failed to grow in the absence of both pyruvate and uridine (Figure 3E). When galactose was substituted for glucose in the growth media, in the absence of either uridine or pyruvate (or both), the cells initially stopped growing and

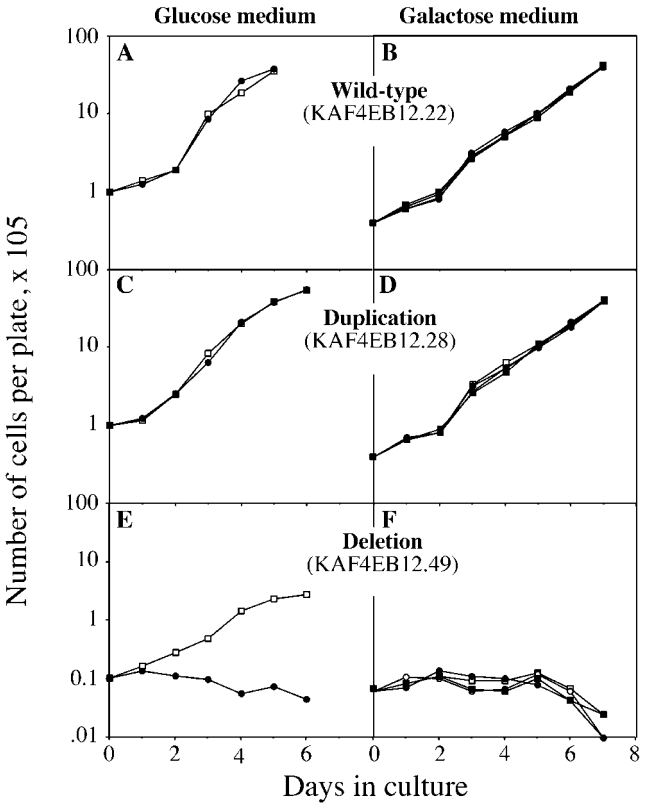

Figure 3. Representative growth curves. The indicated cybrids were grown in high-glucose DMEM in the presence (open squares) or absence (solid circles) of both uridine and pyruvate (A, C, and E), in DMEM (no glucose) plus galactose in the presence (open squares) or absence (solid circles) of both uridine and pyruvate, and in the presence of either uridine (open circles) or pyruvate (solid squares) alone (B, D, and F).

eventually died (they detached from the dishes) (Figure 3F). These growth characteristics are the same as those observed with ρ^0 cells (King and Attardi, 1989).

Levels of mtDNA

We quantitated the steady-state levels of mtDNA in the transmitochondrial cell lines by dot blot hybridization, using known amounts of wild-type mtDNA from HeLa cells as a standard (a representative analysis is shown in Figure 4). As shown in Table 2, cells with wild-type mtDNA (16.6 kb) from patient 1 had a mean mtDNA copy number of 13,056 ± 1063 genomes per cell (n = 20), corresponding to a mass of 0.24 ± 0.02 pg of mtDNA per cell. Cells with duplicated mtDNA (25.3 kb) contained 8550 ± 779 genomes per cell (n = 20), corresponding to 0.24 ± 0.02 pg of mtDNA per cell. The cells harboring dimeric deleted mtDNA (17.6 kb) contained 11,875 ± 1135 molecules per cell (n = 20), corresponding to 0.24 ± 0.02 pg of mtDNA per cell.

These data suggested that cells maintain a constant mass of mtDNA and not a constant number of mtDNA molecules. However, because the dimeric deleted genomes in the deletion cell lines from patient 1 had a molecular mass (17.6 kb) very similar to that of a wild-type mtDNA monomer (16.6 kb), some doubt remained regarding the validity of this conclusion. We therefore examined transmitochondrial cell lines derived from patient 2, containing a different deleted mtDNA (9.1 kb), to investigate further the regulation of mtDNA levels. One wild-type and two deletion cell lines from patient 2 were analyzed. The mtDNA in both 100% deletion cell lines was present only as monomers (i.e., 9.1 kb in size), as determined by *Pvu*II and *Bam*HI restriction enzyme analyses (Figure 1D). The two deletion cell lines contained an average of 23,282 ± 1870 molecules per cell (n = 10) (Table 2), corresponding to 0.23 ± 0.02 pg of mtDNA per cell. The wild-type cell line contained 12,900 ± 1000 molecules per cell (n = 10), corresponding to 0.23 ± 0.02 pg of mtDNA per cell.

Protein Synthesis

The transmitochondrial cell lines from patient 1 were examined for their rates of mitochondrial protein synthesis by labeling cells with a mixture of [^{35}S]methionine and [^{35}S]cysteine in the presence of emetine, an inhibitor of cytoplasmic protein synthesis, for 20 or 30 min. This short period of labeling is expected to reflect the rates of protein synthesis for the mitochondrial translation products (Chomyn, 1996). Four independent protein labelings were performed for each line, and for each labeling, products were analyzed and quantitated in three independent electrophoretic analyses.

A representative protein-labeling analysis is shown in Figure 5. The qualitative patterns of mitochondrial translation products were identical between the duplication and wild-type cell lines. The predicted translation product synthesized from the COX II/Cyt *b* fusion transcript contains the first 99 amino acids (aa) of COX II fused out of frame to the Cyt *b* gene (Figure 5B). The open reading frame in this mRNA encodes a 102-aa polypeptide with a predicted mass of 11.4 kDa. If translated, this fusion polypeptide would be expected to migrate in the gel in the region between ND3 (13.2 kDa) and the doublet of ND4L (10.7 kDa) and ATP 8 (8.0 kDa). However, no unusual polypeptides were observed in this (or any other) region of the gel (Figure 5A).

The rates of translation of each polypeptide, on a "per cell" basis, were essentially the same in duplicated mtDNA lines as in wild-type mtDNA lines (Table 3), irrespective of the number of genomes and genes or the level of transcripts (see below). No mitochondrial translation products were detected in the deleted mtDNA cell lines or in the ρ^0 cell line, as expected.

Analysis of Transcripts

In wild-type mtDNA, all 37 genes are present at one copy per genome. In duplicated mtDNA, the genes residing within the duplicated region are present at two copies per genome, whereas the remaining genes are present at one copy per genome. In deleted dimer mtDNA, the genes located outside the deleted region are present at two copies per genome, whereas the genes located within the deleted region are absent. For our Northern blot analyses, we used probes located inside (ATP 6, COX III, ND3, ND4, ND5,

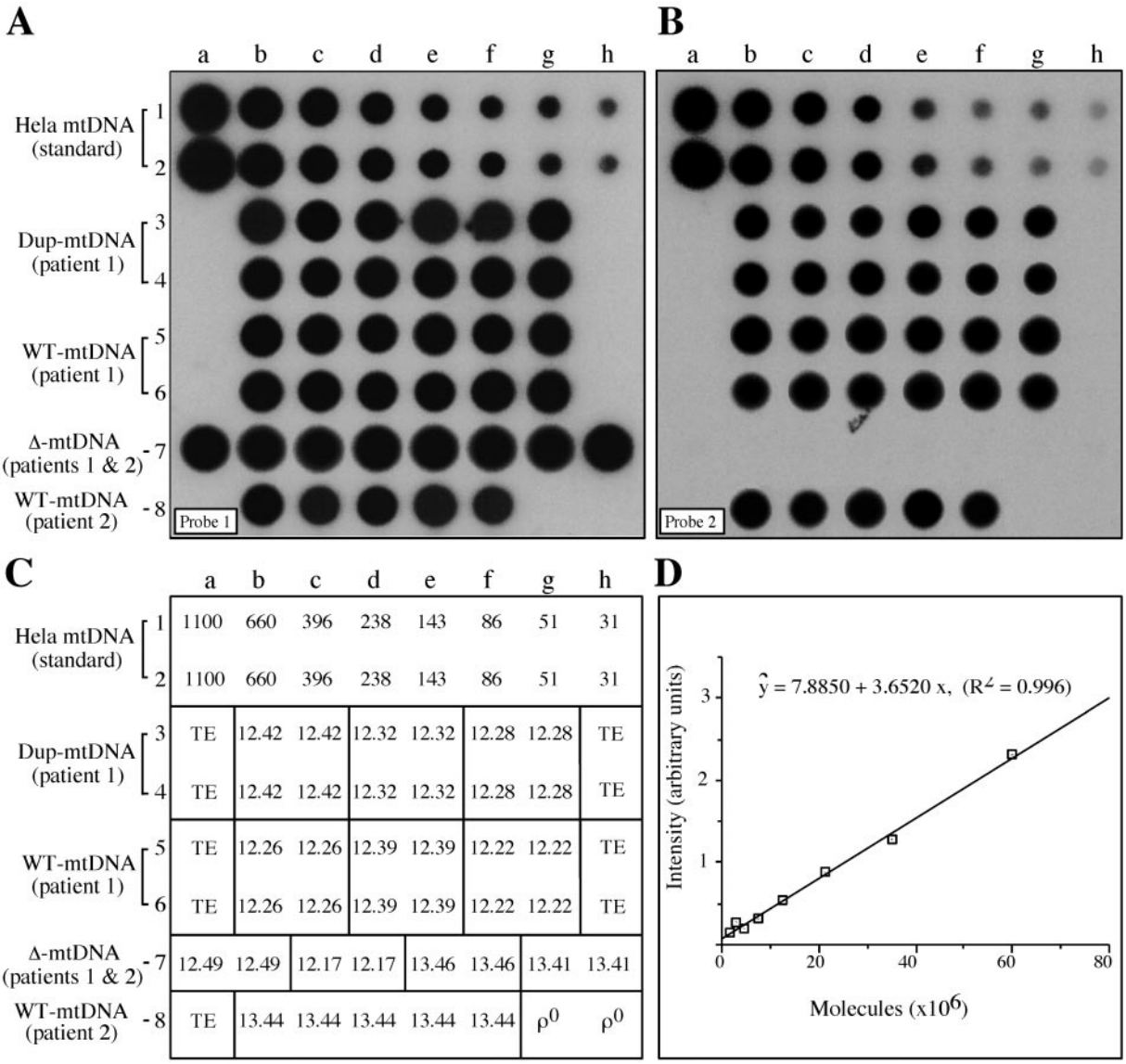

Figure 4. Quantitation of mtDNA. A representative dot blot analysis is shown. Duplicated serial dilutions of purified HeLa cell mtDNA (numbers indicate pg mtDNA spotted) and cell lysates from control ρ^0 cells and from wild-type, duplicated, and deleted lines from patient 1 (cybrid clones12.xx from KAF4EB12; see Table 1) and patient 2 (cybrid clones 13.xx from 206/F4 3.13; see Table 1) were spotted according to the scheme shown in C. Multiple wells identified by the same cell line number denote samples that were collected at different cell passages. Each test well contained DNA from the lysis of 2500 cells. The membrane was hybridized first with probe 2 (B), stripped, and then hybridized with probe 1 (A). The dots were quantitated, and the amount of mtDNA in the various lines was calculated using the standard curve drawn from the HeLa cell data (D). TE, 1× Tris-EDTA buffer control for background.

$tRNA^{Lys}$, and $tRNA^{Glu}$) and outside (12S rRNA, 16S rRNA, ND1, ND2, COX I, $tRNA^{Phe}$, and $tRNA^{Ser(UCN)}$) the deleted region (see Figure 6A). Note that these 14 probes detect not only the three classes of mitochondrial RNA species (rRNAs, tRNAs, and mRNAs) but also transcripts derived from the three major mitochondrial transcription units: the 2.7-kb rRNA region-specific transcription unit, the 16-kb heavy-strand polycistronic transcript, and the 16-kb light-strand polycistronic transcript (Enriquez *et al.*, 1999).

When we analyzed the Northern blot data from patient 1 according to either overall transcript class (rRNA, tRNA, and mRNA) or overall transcription unit (ribosomal, heavy-strand, and light-strand region), we were unable to detect any obvious patterns in the steady-state levels of the transcripts that would allow us to draw meaningful conclusions about transcriptional regulation in these cells. On the other hand, when we analyzed the data according the number of genes present on the respective genomes, detectable patterns emerged.

Table 2. Quantitation of mtDNA copy number in cybrids

Cell line	Patient	bp/genome	Molecules/cell	Picograms/cell
Wild-type				
KAF4EB12.22 (n = 20)	1	16,569	13,050 ± 1,036	0.24 ± 0.02
KAF4EB12.26 (n = 20)	1	16,569	13,118 ± 1,170	0.24 ± 0.02
KAF4EB12.39 (n = 20)	1	16,569	13,000 ± 984	0.24 ± 0.02
Average (KAF4EB12)			13,056 ± 1,063	0.24 ± 0.02
206/F4 3.13.44 (n = 10)	2	16,569	12,900 ± 1,000	0.23 ± 0.02
Duplication				
KAF4EB12.28 (n = 20)	1	25,323	8,702 ± 803	0.25 ± 0.02
KAF4EB12.32 (n = 20)	1	25,323	8,470 ± 791	0.24 ± 0.02
KAF4EB12.42 (n = 20)	1	25,323	8,478 ± 742	0.24 ± 0.02
Average (KAF4EB12)			8,550 ± 779	0.24 ± 0.02
Deletion				
KAF4EB12.17 (D)[a] (n = 20)	1	17,508	11,810 ± 1,083	0.23 ± 0.02
KAF4EB12.49 (D) (n = 20)	1	17,508	11,939 ± 1,187	0.23 ± 0.02
Average (KAF4EB12)			11,875 ± 1,135	0.23 ± 0.02
206/F4 3.13.41 (M) (n = 10)	2	9,049	22,854 ± 1,874	0.22 ± 0.02
206/F4 3.13.46 (M) (n = 10)	2	9,049	23,710 ± 1,866	0.23 ± 0.02
Average (206/F4 3)			23,282 ± 1,870	0.23 ± 0.02

[a] D, dimeric mtDNA; M, monomeric mtDNA.

Transcription in Duplication Cell Lines. On an absolute basis (i.e., transcripts per cell), the steady-state levels of the seven "1-copy" gene transcripts were decreased in the duplication cell lines compared with those in wild-type lines (by an average of 65%; range, 45–87%; Table 4A, column A). Because the total number of 1-copy genes per cell was 66% of the wild-type value (i.e., 8550/13,056 bp; Table 4A, e/d), one would have predicted that if transcription in duplication lines were regulated in the same way as in wild-type lines, the number of 1-copy gene transcripts in duplication lines should have been 66% of the wild-type value, which agrees well with the average of 65%. For the "2-copy" genes, one would have predicted that the level of these transcripts in duplication lines should have been 132% of the wild-type values (i.e., 2 × 66%). In fact, six of the seven examined transcripts were well below this value (range, 64–107%; Table 4B, column A); only $tRNA^{Ser(UCN)}$ had the predicted value (i.e., 131%). The same pattern was also observed when transcription was measured on a per-gene basis (Table 4B, column C).

Transcription in Deletion Cell Lines. As expected, we observed no transcripts from genes located inside the deleted region in deletion dimer cell lines. Because the number of deleted mtDNAs in deletion dimer lines was only 91% of the number of wild-type mtDNAs (i.e., 11,875/13,056 bp; Table 2), the per-cell number of 2-copy genes was 182% of the wild-type gene number (i.e., 2 × 91%). Thus, the level of transcripts in deletion dimer lines (i.e., transcripts per cell) should have been 182% of the wild-type values. In fact, only two of the seven examined transcripts, ND1 and ND2, had this expected value (Table 4B, column B). The remaining five transcripts were either significantly decreased (average of 46% for 12S rRNA and COX I; $p < 0.05$) or mildly reduced (average of 80% for 16S rRNA, $tRNA^{Phe}$, and $tRNA^{Ser(UCN)}$). The per-gene levels of the latter five transcripts were also reduced, both when compared with the levels in wild-type lines (range, 18–51%; Table 4B, column D) and in duplication lines (range, 38–68%; Table 4B, column E).

Transcription of Genes Spanning the Deletion Breakpoint. The rearrangement breakpoint in patient 1 joins the first 297 bp of the COX II gene to the last 192 bp of the Cyt *b* gene. If cleavage of the COX II/Cyt *b* fusion gene in the polycistronic precursor RNA occurred normally (i.e., at $tRNA^{Asp}$ at the 5′ end of COX II and at $tRNA^{Thr}$ at the 3′ end of Cyt *b*; Ojala *et al.*, 1981), a transcript of 489 nt (excluding polyadenylation) would be expected (Figure 6A). We designed probes to distinguish the transcripts arising from the authentic COX II (709 nt) and Cyt *b* (1145 nt) genes from that derived from the fusion gene (489 nt). Using the COX II-in probe, we detected only the authentic COX II transcript in both wild-type and duplication lines, as expected (Figure 6B). Using the COX II-out and Cyt *b*-out probes, we detected not only the authentic COX II and Cyt *b* transcripts in wild-type and duplication lines but a hybridizing band migrating at ~0.5 kb as well, consistent with the expected size of the fusion transcript, in duplication and deletion lines (Figure 6B; also see Nakase *et al.*, 1990). No fusion gene transcript was present in wild-type lines, and no authentic COX II or Cyt *b* transcripts were present in deleted lines, as expected.

As with the other 1-copy genes in duplication lines (see above), the number of authentic COX II, authentic Cyt *b*, and COX II/Cyt *b* fusion transcripts should have been 66% of the wild-type value. The steady-state level of the authentic COX II transcript was 79% of the level in wild-type lines (i.e., average of 71% with the COX II-in probe and 87% with the COX II-out probe), and that of the authentic Cyt *b* transcript was 77% of the wild-type value (Table 5, row A), which agrees well with the prediction of 66%.

Even though the per-cell number of fusion genes present in the duplicated lines was equal to that of the authentic COX II and Cyt *b* genes, the steady-state level of the fusion

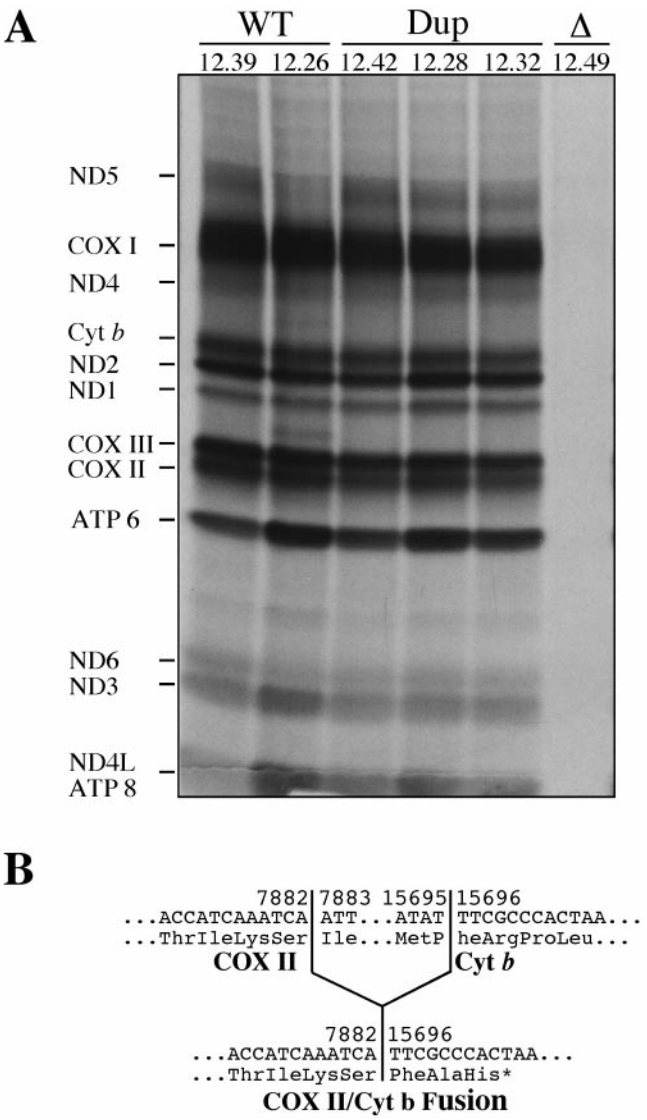

Figure 5. Mitochondrial translation in representative homoplasmic cybrid clones (notation as in Figure 2). (A) Fluorogram of mitochondrial translation products. The predicted mtDNA-encoded polypeptides are shown at left. (B) Nucleotide and deduced amino acid sequences across the rearrangement breakpoint straddling the authentic COX II and Cyt *b* genes (see Figure 1). Numbers denote map positions inside and outside the deleted region. Note that the conceptual translation of the portion of the fusion mRNA located beyond the breakpoint is out of frame, resulting in a premature termination codon.

mRNA was lower than that of authentic COX II mRNA (i.e., 62%, using the COX II-out probe) but was higher than that of authentic Cyt *b* (i.e., 149%, using the Cyt *b*-out probe) (Table 5, row B). Similarly, the level of fusion transcripts in duplication lines was lower than that of the authentic COX II transcripts in wild-type lines (54%) but was higher than that of the authentic Cyt *b* transcripts in wild-type lines (115%) (Table 5, row C). Likewise, the level of the fusion transcript in deletion lines was lower than that of COX II (88%) but higher than that of Cyt *b* (135%) (Table 5, row D) in wild-type lines.

Table 3. Protein synthesis

	Translation/cell Ratio (Dup:WT)
1-copy genes	
ATP 6	1.10 ± 0.25
ATP 8	1.02 ± 0.16
COX III	0.86 ± 0.18
ND3	0.83 ± 0.10
ND4	0.89 ± 0.11
ND5	0.92 ± 0.09
ND6	0.92 ± 0.08
2-copy genes	
ND1	1.04 ± 0.19
ND2	1.18 ± 0.31
COX I	0.85 ± 0.12
Break point genes	
COX II (authentic)	0.84 ± 0.20
Cyt *b* (authentic)	0.96 ± 0.20
COX II/Cyt *b* fusion	Not detected

DISCUSSION

Mechanisms of mtDNA Maintenance

The most striking result obtained here was that the mtDNA copy number in homoplasmic transmitochondrial cells containing wild-type, duplicated, and deleted genomes was inversely proportional to the size of the respective mtDNAs (Table 2). These results imply that transformed human osteosarcoma cells (and perhaps mammalian cells in general) maintain a constant mass of mtDNA and not a constant number of mitochondrial genomes.

The ability of a cell to titrate its mass of mtDNA was discovered in yeast almost 30 years ago (Fukuhara, 1969; Hollenberg *et al.*, 1972; Nagley and Linnane, 1972; for review, see Dujon, 1981). Specifically, partially deleted mtDNA subgenomic circles in petite ρ^- yeast strains were amplified at the expense of the wild-type genomes, resulting in a number of ρ^- genomes corresponding in mass to the equivalent number of full-length wild-type genomes that they had displaced. Similar observations have also been reported for mouse cells: when hybrid and cybrid progeny lines were constructed between mouse LA9 cells containing almost exclusively mtDNA monomers and mouse LDTK cells containing only mtDNA dimers, approximately equal mass proportions of both parental mtDNAs were maintained in most progeny lines (Howell *et al.*, 1984).

The mechanism by which mtDNA levels were regulated in our cells is unknown. Regulation of mtDNA copy number is closely related to the regulation of mtDNA replication, which initiates at the L-strand promoter of transcription (for review, see Taanman, 1999), and Moraes *et al.* (1999) have provided evidence that one or more replication factors could be rate limiting for the maintenance of mtDNA copy number in human cells. The components of the mtDNA replication machinery, all of which are encoded by nuclear genes (for review, see Shadel and Clayton, 1997), are obvious potential

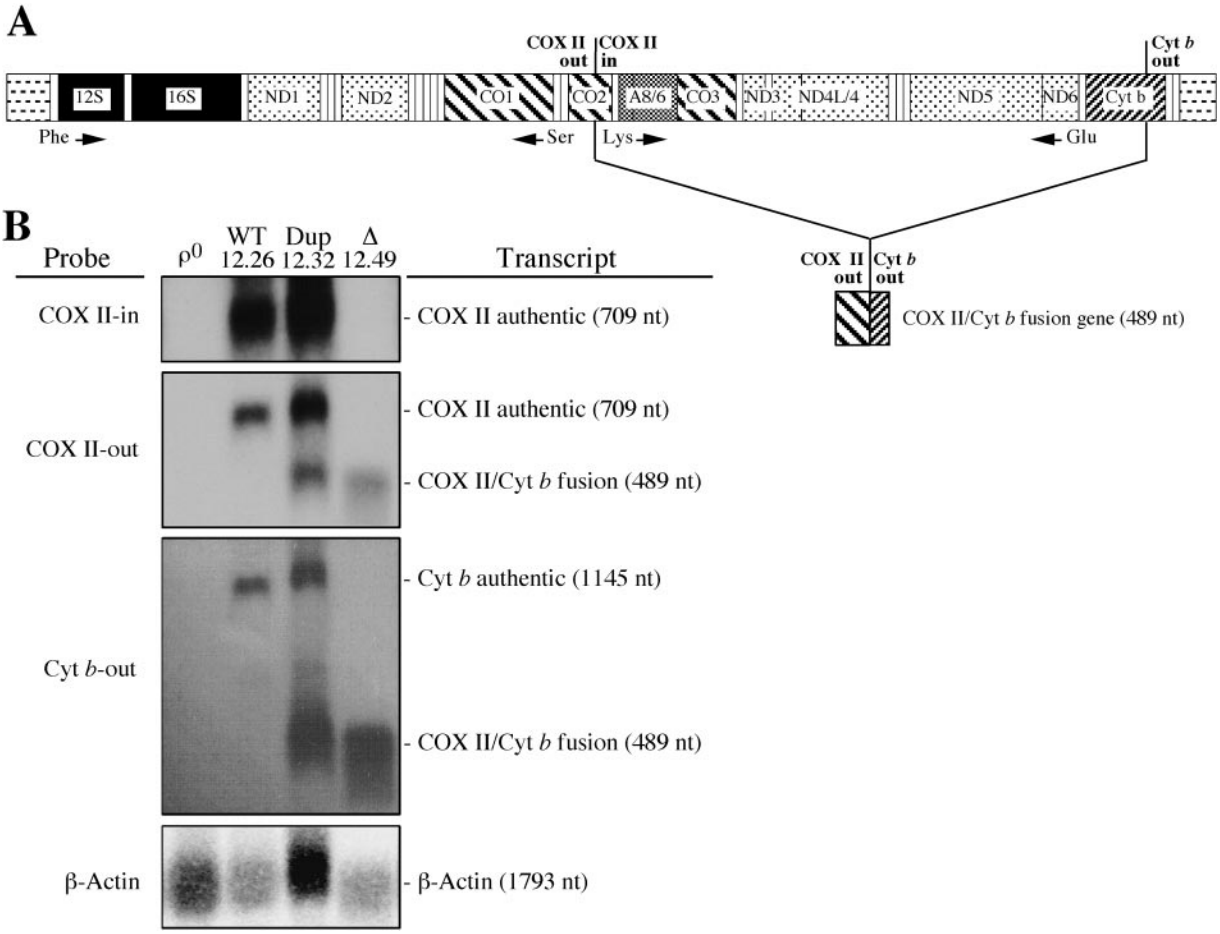

Figure 6. Northern blot analyses. (A) Linearized map of wild-type mtDNA. The COX II/Cyt *b* fusion gene and the predicted size of its transcript, are shown below the map, as are the in and out probes (bold) used to detect the authentic and fusion mRNAs. The four tRNAs examined in this study, and the direction of their transcription (leftward, L-strand encoded; rightward, H-strand encoded), are also shown. (B) Northern blots of representative cybrid lines (notation as in Figure 2) showing transcription in the breakpoint region, using the in and out probes. The expected sizes of the indicated transcripts are shown at right.

candidates for this role. Among the currently known proteins associated with mtDNA replication are mitochondrial DNA polymerase γ (pol γ), RNA polymerase, mitochondrial transcription factor A (mtTFA), RNA-processing enzymes, factors that bind to termination-associated sequences, topoisomerases, and single-stranded DNA-binding protein (SSB). Of these, the influence of pol γ, mtTFA, and SSB on mtDNA copy number has been studied. Although the levels of mtTFA (Larsson *et al.*, 1994, 1998; Poulton *et al.*, 1994) and SSB (Schultz *et al.*, 1998), but not of pol γ (Davis *et al.*, 1996; Schultz *et al.*, 1998), have been correlated with copy number, cause and effect have not been demonstrated.

We do not think that the number of replication origins present on each genome (Bogenhagen and Clayton, 1976) influenced the copy number of mtDNA in our cell lines. For patient 1, each deleted mtDNA dimer contains two origins of heavy-strand replication (O_H) and two origins of light-strand replication (O_L), twice the number found in wild-type mtDNA (see Figure 1A). However, the copy number was very similar between cells harboring these two mtDNA types. Similarly, the corresponding duplicated mtDNA also contains two O_Hs and two O_Ls, yet cells with duplicated genomes had a copy number significantly lower than the copy number found in wild-type cells. For patient 2, each deleted mtDNA contains one O_H and one O_L, the same as in wild-type mtDNA, yet the number of genomes in the deletion cells was significantly higher. If the number of replication origins had influenced the cellular mtDNA copy number, we should have found that the number of mtDNAs per cell corresponded to the numbers of origins. In fact, we observed no such relationship.

Regulation of the initiation of mtDNA replication is probably a necessary, but not sufficient, step for the maintenance of mtDNA mass, and other steps in the replication program (e.g., extension and resolution of daughter strand molecules) may come into play. For example, the size of the organellar nucleoside pools could be rate limiting for mtDNA replication (Bogenhagen *et al.*, 1981; Bestwick *et al.*, 1982; Lecrenier and Foury, 1995). The tight control of mtDNA mass could also result from an equally tight control over mitochondrial

Table 4. Quantitation of transcripts

	Genes/genome[a]			Genes/cell			Transcripts/cell		Transcripts/gene/cell		
	WT a	Dup b	Del c	WT d	Dup e	Del f	Dup/WT A	Del/WT B	Dup/WT C = (Ad)/e	Del/WT D = (Bd)/f	Del/Dup E = D/C
A. 1-copy genes											
$tRNA^{Lys}$	1	1	0	13,056	8,550	0	0.55[b]	0	0.84	–	–
ATP 8/ATP 6	1	1	0	13,056	8,550	0	0.83	0	1.27	–	–
COX III	1	1	0	13,056	8,550	0	0.87[b]	0	1.33	–	–
ND3	1	1	0	13,056	8,550	0	0.60	0	0.92	–	–
ND4L/ND4	1	1	0	13,056	8,550	0	0.45[b]	0	0.69	–	–
ND5	1	1	0	13,056	8,550	0	0.51	0	0.78	–	–
$tRNA^{Glu}$	1	1	0	13,056	8,550	0	0.73	0	1.11	–	–
Average							0.65	0	0.99	–	–
Predicted value[c]							0.66	0	1.00	–	–
B. 2-copy genes											
ND1	1	2	2	13,056	17,100	23,750	0.82	1.86[b]	0.62	1.02	1.64
ND2	1	2	2	13,056	17,100	23,750	1.05	1.77[b]	0.80	0.97	1.21
COX I	1	2	2	13,056	17,100	23,750	0.64[b]	0.32[b]	0.48	0.18	0.38
12S rRNA	1	2	2	13,056	17,100	23,750	0.71	0.59[b]	0.54	0.32	0.59
16S rRNA	1	2	2	13,056	17,100	23,750	0.78	0.73	0.59	0.40	0.68
$tRNA^{Phe}$	1	2	2	13,056	17,100	23,750	1.07	0.74	0.82	0.41	0.50
$tRNA^{Ser(UCN)}$	1	2	2	13,056	17,100	23,750	1.31[b]	0.93	1.00	0.51	0.51
Average							0.91	0.99	0.69	0.54	0.79
Predicted value[c]							1.32	1.82	1.00	1.00	1.00

[a] Genomes per cell: 13,056 in monomeric wild-type (WT) lines; 8550 in duplication (Dup) lines; 11,875 in deletion dimer (Del) lines.

[b] Significant difference compared with wild-type value ($p < 0.05$).

[c] Assumes that transcription in rearranged lines is regulated in the same way as in wild-type lines. Thus, for 1-copy genes, transcription in Dup lines is 1× (8,550/13,056) relative to WT lines. For 2-copy genes, transcription in Dup lines is 2× (8,550/13,056) and in Del dimer lines is 2× (11,875/13,056) relative to WT lines.

dNTP pools (Reichard, 1988; Zhao *et al.*, 1998). The finding that mutations in thymidine phosphorylase (a cytosolic enzyme that converts thymidine to thymine) causes depletions and multiple large-scale deletions of mtDNA in patients with mitochondrial neurogastrointenstinal encephalomyopathy (Nishino *et al.*, 1999), supports this possibility. Thus, we view the regulation of mtDNA copy number as a multistep process, including events associated with initiation of DNA synthesis (i.e., the recognition by *trans*-acting factors of *cis*-acting target elements) and the incorporation of dNTPs. This multistep regulation event also responds to external stimuli, such as thyroid hormone (Enriquez *et al.*, 1999), or to intracellular ADP/ATP ratios (Murdock *et al.*, 1999).

Table 5. Quantitation of transcripts in the break point region

	Transcripts/cell		
	Predicted value[a]	COX II	Cyt *b*
COX II-in probe			
Authentic in Dup/Authentic in WT	0.66	0.71	
COX II-out and Cyt b-out probes			
A. Authentic in Dup/Authentic in WT	0.66	0.87	0.77
B. Fusion in Dup/Authentic in Dup	1.00	0.62	1.49
C. Fusion in Dup/Authentic in WT	0.66	0.54	1.15
D. Fusion in Del/Authentic in WT	1.82	0.88	1.35
E. Fusion in Dup/Fusion in Del (=C/D)	0.36	0.61	0.86

[a] See footnote in Table 4.

Regulation of mtDNA Gene Expression

The steady-state levels of mtDNA-encoded RNAs detected in duplication, deletion, and wild-type cells suggest that gene copy number influences the levels of mature transcripts. Wild-type mtDNA encodes three polycistronic transcription units, two derived from the heavy strand (2.7 and 16 kb long) and one from the light strand (16 kb long). We presume that in the duplicated mtDNA from patient 1 there are six polycistronic transcription units, of which two, both 8.8 kb long, are novel, because they are derived from the heavy and light strands of the duplicated region and contain RNAs derived from the fusion gene. We found that on a per-cell basis, the steady-state level of the 1-copy gene transcripts in duplication lines was reduced by approximately one-third relative to that in wild-type lines, but on a per-gene basis, the number of transcripts was essentially the same in both cell lines. This implies that there was no obvious up- or down-regulation of the steady-state number of 1-copy transcripts in duplicated lines. For the 2-copy gene transcripts, however, transcription on a per-gene basis was reduced for six of the seven examined genes, compared with that in wild-type, implying that the steady-state level of the

2-copy transcripts in duplicated lines was indeed regulated. Our studies, however, were not able to address whether this regulation was due to altered rates of transcription, to changes in turnover, or to both. Based on the 1-copy transcript data, we believe that regulation of the 2-copy transcripts would be more compatible with a post-transcriptional mechanism.

We presume that there are six polycistronic transcription units synthesized from the deleted dimer mtDNA in patient 1 (i.e., two 2.7-kb ribosomal transcriptional units and four novel 8.8-kb units, two derived from the heavy strand and two from the light-strand; see Figure 1A). We found that the steady-state levels of five of the seven examined transcripts in deleted dimer lines were decreased (on both a per-cell and per-gene basis) not only when compared with wild-type lines but also when compared with duplicated lines, with the notable exception of the two ND transcripts (Table 4). We do not know why the levels of ND1 and ND2 did not decrease in a corresponding manner. Nevertheless, the overall results imply that, as in duplicated lines, transcription of 2-copy genes in deleted dimer lines is also regulated post-transcriptionally but with a much greater degree of RNA turnover than in duplicated lines. We believe that the "looser" regulation of transcription in deleted dimer lines is due to the fact that translation is not occurring correctly (or is not occurring at all) in these cells (note that four of the five transcripts that were examined [two rRNAs and two tRNAs] are involved directly in the translation process).

As with the other 1-copy gene transcripts in duplication lines, the steady-state levels of the authentic COX II and Cyt *b* transcripts, on a per-gene basis, were the same in both wild-type and duplication lines and were apparently unrelated to the presence of the COX II/Cyt *b* fusion transcript (which was present only in the duplication lines). In addition, the amount of COX II/Cyt *b* fusion transcript appeared to be unrelated to the amount of the corresponding authentic genes (i.e., the level of fusion transcript was much lower than that of the COX II transcript but much higher than that of the Cyt *b* transcript). These results suggest that the presence of the fusion gene transcript exerts little influence on the steady-state levels of the related authentic transcripts. We believe, however, that only the comparison with the authentic COX II transcript is biologically meaningful. This is because both the authentic COX II and the COX II/Cyt *b* fusion transcripts have the same 5′ ends and probably engage the mitochondrial translational machinery in the same way. If true, these two transcripts would likely compete with each other for entry into any particular ribosome. On the other hand, there is no such competition between the COX II/Cyt *b* fusion transcript and the authentic Cyt *b* transcript, because they do not share the same 5′ end.

The ability of the cell to adjust the steady-state levels of transcripts in duplicated mtDNAs according to the number of genes present was also observed with translation, such that the rate of translation in duplication cells was essentially identical to the rate in wild-type cells. Thus, there appears to be an interplay between transcription and translation in modulating the respiratory requirements of the cell. The results reported here and elsewhere (Larsson *et al.*, 1998; for review, see Turnbull and Lightowlers, 1998) indicate that the level of mtDNA does not necessarily correlate with the level of mtDNA-encoded polypeptides or even with the steady-state level of mitochondrial RNAs, suggesting that mtDNA copy number is not normally rate limiting for mitochondrial function.

The potential interplay between transcription and translation, of course, does not apply to the deletion lines, in which the translational machinery is impaired constitutively and no authentic COX II or Cyt *b* transcripts are made. Relative to the level of fusion transcripts in duplication lines, the level in deletion lines was much lower than expected (Table 5, row E). We interpret this result to mean that there was a paucity of transcripts in deletion lines because the mRNAs were rapidly degraded in the absence of full-length translation. Despite the fact that we were unable to detect a fusion polypeptide in duplication lines (Figure 5), the relatively higher level of stable fusion mRNAs in these lines implies that they were translatable across their entire length (Hayashi *et al.*, 1991) and were therefore less susceptible to degradation.

Implications for Pathogenesis

We have shown here that transmitochondrial cells harboring the deleted mtDNA found in patient 1 were severely deficient in respiratory chain activity, presumably because of the absence of mitochondrial protein translation resulting from the loss of essential tRNA genes from the deleted mitochondrial genome. On the other hand, cells containing the corresponding duplicated mtDNA showed no obvious phenotypic defects, even though they contained mtDNAs with the identical rearrangement breakpoint present in the deleted genome. Protein translation in duplicated lines was normal, despite the presence of extra copies of mtDNA genes in the duplication region. Taken together, these results support the "missing tRNA hypothesis" for the pathogenesis of deleted mtDNAs (Nakase *et al.*, 1990) and are consistent with previous observations in cybrids regarding deleted (Hayashi *et al.*, 1991) and duplicated mtDNAs (Holt *et al.*, 1997). They are also consistent with morphological analyses of skeletal muscle from heteroplasmic patients harboring deletions (Mita *et al.*, 1989; Moraes *et al.*, 1992) and/or duplications (Manfredi *et al.*, 1997), which showed that COX-deficient fibers contained mainly or exclusively deleted mtDNAs, whereas the duplicated mtDNAs were present in morphologically normal fibers. We also do not think that an imbalance of tRNAs or mRNAs is of primary importance in the potential pathogenicity of duplicated mtDNAs, because the interplay between transcription and translation tends to "smooth out" the effects of the imbalance in the number of genes on the duplicated circle (King and Attardi, 1993).

Although the duplicated mtDNA in patient 1 was apparently not pathogenic, this conclusion may not be generalizable to other duplications, because different duplication breakpoints generate different fusion genes, some of which could specify novel proteins with novel effects. For example, the large-scale mtDNA gene rearrangement in maize that gives rise to the *urf13* gene causes cytoplasmic male sterility in plants (Dewey *et al.*, 1988; Rhoads *et al.*, 1994). In our particular case, we found no evidence that the COX II/Cyt *b* fusion protein was present in duplicated lines from patient 1, perhaps because it was not synthesized. Alternatively, the fusion polypeptide may have been synthesized (because both the fusion transcript and all required tRNAs were present; Hayashi *et al.*, 1991) but was then degraded rapidly.

In the latter case, the absence of observable amounts of the 102-aa fusion polypeptide (of which 99 are derived from the N terminus of COX II) implies, first, that it was not incorporated into complex IV and, second, that there was no dominant effect of the fusion protein on respiratory chain function.

Duplicated mtDNAs, although they may not be pathogenic in and of themselves, could still have pathogenic consequences if they are recombination intermediates that could give rise to deleted mtDNAs (Poulton *et al.*, 1993, 1995), which are pathogenic. This is particularly relevant to those disorders in which duplicated mtDNAs, but not the corresponding deleted mtDNAs, have been detected, such as maternally inherited diabetes and deafness (Dunbar *et al.*, 1993). It will be useful to see whether long-term culture of 100% duplicated mtDNA cybrid lines can generate the corresponding deleted mtDNAs, presumably via recombination, as is the case in yeast (Evans and Clark-Walker, 1985; Clark-Walker, 1989; Shaw *et al.*, 1989). Such a finding not only would clarify the relationship between these two rearranged mtDNAs but also might shed light on the mechanism of their generation.

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