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A Novel Mitochondrial DNA-like Sequence in the Human Nuclear Genome

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We describe here a nuclear mitochondrial DNA-like sequence (numtDNA) that is nearly identical in sequence to a continuous 5842 bp segment of human mitochondrial DNA (mtDNA) that spans nucleotide positions 3914 to 9755. On the basis of evolutionary divergence among modern primates, this numtDNA molecule appears to represent mtDNA from a hominid ancestor that has been translocated to the nuclear genome during the recent evolution of humans. This numtDNA sequence harbors synonymous and nonsynonymous nucleotide substitutions relative to the authentic human mtDNA sequence, including an array of substitutions that was previously found in the cytochrome *c* oxidase subunit 1 and 2 genes. These substitutions were previously reported to occur in human mtDNA, but subsequently contended to be present in a nuclear pseudogene sequence. We now demonstrate their exclusive association with this 5842-bp numtDNA, which we have characterized in its entirety. This numtDNA does not appear to be expressed as a mtDNA-encoded mRNA. It is present in nuclear DNA from human blood donors, in human SH-SY5Y and A431 cell lines, and in ρ^0 SH-SY5Y and ρ^0 A431 cell lines that were depleted of mtDNA. The existence of human numtDNA sequences with great similarities to human mtDNA renders the amplification of pure mtDNA from cellular DNA very difficult, thereby creating the potential for confounding studies of mitochondrial diseases and population genetics. © 1999 Academic Press

INTRODUCTION

The appearance of nuclear DNA sequences with homology to mitochondrial genes is a common occurrence in most organisms (Arctander, 1995; Blanchard and Schmidt, 1996; Corral *et al.*, 1989; Hadler *et al.*, 1983; Kocher *et al.*, 1989; Lopez *et al.*, 1994; Sunnucks and Hales, 1996). Almost all regions of the human mitochondrial genome are represented by homologous nuclear DNA sequences (Collura and Stewart, 1995; Fukuda *et al.*, 1985; Hu and Thilly, 1995; Nomiyama *et al.*, 1984, 1995; Shay and Werbin, 1992; Tsuzuki *et al.*, 1983; Zischler *et al.*, 1995). Typically, these nuclear mitochondrial DNA-like sequences (numtDNA) have been reported as short gene fragments that are less than 600 bp in size and that show varying degrees of similarity with short stretches of mitochondrial DNA (mtDNA). Multiple noncontiguous fragments of mtDNA-like sequences are occasionally end-joined and inserted into nuclear DNA sequences (Lopez *et al.*, 1994; Shay and Werbin, 1992). In plants and yeast, these sequences have been localized to introns, flanking nuclear genes, and telomeric regions (Louis and Haber, 1991; Pichersky and Tanksley, 1988), but the exact localization of numtDNA sequences has been studied only rarely in humans (Shay and Werbin, 1992; Zischler *et al.*, 1995).

Both the mechanisms and the functional consequences that underlie the interorganelle transposition of DNA sequences are unknown. Interorganelle DNA transpositions may arise either from continuous ongoing processes that are associated with cellular mutagenesis or cellular catastrophes (Corral *et al.*, 1989) or from single DNA transfers of ancestral significance. Once transferred to the nucleus, these numtDNA sequences appear to evolve at the much slower rate of nuclear DNA (Collura and Stewart, 1995). Phylogenetic analyses indicate that many of these numtDNA sequences were transferred from the mitochondria to the nucleus long before the origin of modern humans (Collura and Stewart, 1995; Fukuda *et al.*, 1985; Hu and Thilly, 1994), while others indicate more recent transfers of mtDNA sequences to the nucleus (Wallace *et al.*, 1997; Zischler *et al.*, 1995). If these numtDNA sequences did not incur much DNA damage prior to transfer, they represent fossilized signatures of ancestral mtDNA, and they are of significance from an evolutionary perspective.

Previously, in the course of searching for genetic

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changes in mtDNA that associate with Alzheimer disease, sequences for the cytochrome *c* oxidase subunit 1 and 2 genes (COI, COII) that were nearly identical (98%) to those in human mtDNA were found (Davis *et al.*, 1997). These two gene sequences were thought to be mitochondrial genes that carried nucleotide substitutions, and they were demonstrated to be present at elevated levels in Alzheimer disease patients compared to normal control individuals using a competitive primer extension assay. The presence of these substitutions in mtDNA was subsequently questioned (Hirano *et al.*, 1997; Wallace *et al.*, 1997). Indirect evidence of a nuclear pseudogene was provided that was based exclusively on the ability to PCR amplify the COI and COII containing fragments in cell lines presumed to be depleted of all mitochondrial DNA. However, even a single copy of a mitochondrial genome that contained this sequence would obviate those results.

To address rigorously the question of nuclear localization of mitochondrial-like sequences, we attempted to provide definitive evidence that these sequences are not contained in mitochondria. In this paper, we describe for the first time the complete sequence of an approximately 5.8-kb fragment of contiguous mtDNA-like sequence, with greater than 98% DNA sequence identity to modern human mtDNA. This sequence is flanked by nonmitochondrial DNA sequences, does not appear to be expressed as polyadenylated mRNA that originated in the mitochondria, and is not present in purified mitochondria from human brain tissue or immunopurified mitochondria from human SH-SY5Y cells. The data presented in this paper firmly establish that this mtDNA-like sequence is of nonmitochondrial origin and is embedded in nuclear DNA on chromosome 1. As suggested by Wallace *et al.* (1997) on the basis of small fragments of this molecule, we provide additional evidence that this 5842-bp mtDNA-like sequence could represent the translocation of mtDNA from an early, hominid ancestor, followed by its fixation in the nuclear genome during subsequent evolution. Thus far, this human numtDNA sequence is the longest described in its entirety, and it represents the most recent nuclear insertion of undamaged mtDNA reported to date.

MATERIALS AND METHODS

Bacterial artificial chromosome library. A human genomic library that consists of lymphocyte DNA partially digested with *Hin*dIII restriction endonuclease and inserted into the pBeloBAC11 vector (Genome Systems, Inc., St. Louis, MO), with insert sizes ranging from 50 to over 240 kb, was screened by PCR (Mullis and Faloona, 1987; Saiki *et al.*, 1988). Primers used for PCR amplification were designed to select for COI gene sequences that contain substitutions relative to the Cambridge Reference Sequence (CRS) of human mtDNA (Anderson *et al.*, 1981) at nucleotide positions 6366 and 7146 (Davis *et al.*, 1997; Wallace *et al.*, 1997). Accordingly, primer sequences were complementary to wildtype mtDNA (Anderson *et al.*, 1981) but contained G → A and T → C substitutions, respectively, at the 3′-ends (L-strand primer, 5′-CCTTACACCTAGCAGGTA; H-strand primer, 5′-ACGCCGATGAATATGATAGC). One positive clone was identified out of approximately 110,000 clones screened (Genome Systems, Inc.).

DNA sequencing. BAC clonal DNA was purified with Magnum KB-100 Columns (Genome Systems, Inc.). The numtDNA region of the clonal insertion was sequenced in its entirety by direct sequencing of PCR products amplified from the insertion and by subcloning and sequencing fragments of the insertion.

An overview of the sequencing strategy is depicted in Fig. 1. Nine portions of the insert were amplified with internal PCR primers complementary to the CRS (Anderson *et al.*, 1981). The 9 PCR products spanned nucleotide positions 3908 to 6947, 3908 to 4798, 4699 to 7472, 5009 to 5351, 5234 to 7834, 5596 to 6699, 6657 to 7193, 7785 to 8287, and 8572 to 9041. The PCR products were purified (QIAquick PCR Purification Kit, Qiagen, Valencia, CA) and sequenced using primer sequences complementary to the ends or internal sequences of the amplification products and the Prism DyeDeoxy Terminator chemistry (Perkin–Elmer, Foster City, CA).

The BAC clone DNA was restricted with *Ssp*I and *Bsp*120I and separated by horizontal agarose gel electrophoresis. A 1.1-kb band and a 1.0-kb band, respectively, were excised from the gel and subcloned into the pBC phagemid cloning vector (Stratagene, San Diego, CA) using XL1Bue competent cells (Stratagene). Recombinant clones were selected, and plasmid DNA was purified (Wizard Plus Series 9600 Miniprep Reagent System, Promega, Madison, WI) and sequenced using T7 and Sp6 primers and Prism DyeDeoxy Terminator chemistry (Perkin–Elmer).

To obtain sequence data at the borders of the numtDNA fragment and the adjacent nonmitochondrial DNA, BAC DNA was sequenced using the BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer) (Lee *et al.*, 1997) and primers complementary in sequence to ND1 and COXIII sequences (heavy strand primer at nucleotide position 3966, 5′-CGGCTATGAAGAATAGGCGA; light strand primers at nucleotide positions 9245 and 9673, 5′-TCACATGCCTATCATATAGTA and 5′-GAAACCAAATAATTCAAGCACTGC).

Sequencing reaction products were purified by ethanol precipitation or with CentriSep spin columns (Princeton Separations, Adelphia, NJ) and were separated by electrophoresis using the Applied Biosystems Model 373A DNA sequencing system (Applied Biosystems Perkin–Elmer). The Sequence Navigator software (Applied Biosystems Perkin–Elmer) was used for analysis of sequence data (GenBank Accession No. AF134583), and mutations were identified by comparison with the CRS (Anderson *et al.*, 1981).

DNA isolation from blood cells. Blood samples were collected in EDTA vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) and kept at 4°C for no longer than 24 h. The platelet/white blood cell fraction was isolated with Accuspin Tubes (Sigma Diagnostics, St. Louis, MO) using the following procedure. Three milliliters of HISTOPAQUE 1077 was added to the upper chamber of Accuspin Tube, and the device was centrifuged at 1000*g* for 30 s. Two to three milliliters of blood was then introduced into the upper chamber and separated by centrifugation at 1000*g* for 10 min at room temperature. After centrifugation, the plasma and white blood cell layers were transferred to a new tube, and the white blood cells were sedimented by centrifugation at 7000*g* for 10 min. The white cell pellet was resuspended in 0.4 ml of a solution containing 0.9% sodium chloride/1 mM EDTA and stored at −80°C.

Frozen white blood cells (0.2 ml) were thawed and then sedimented by centrifugation at 12,000*g* for 5 min. The white cell pellet was washed twice with 0.6 ml of Dulbecco's phosphate-buffered saline (PBS; Gibco BRL, Gaithersburg, MD) and resuspended in 0.2 ml of water. The cells were lysed by incubation in a 100°C water bath for 10 min. After cooling to room temperature, the cellular debris was sedimented by centrifugation at 14,000*g* for 2 min. The supernatant was transferred to a new vial, and the approximate concentration of the crude DNA preparation was estimated by UV absorption at 260 nm. The DNA samples were stored at −80°C.

Cells and cultures. Reagents for tissue culture were purchased from Gibco BRL. All other reagents were from Sigma Chemical Co. (St. Louis, MO). SH-SY5Y human neuroblastoma cells and A431

human carcinoma cells were maintained as described (Miller *et al.*, 1996). ρ^0 118/5 and ρ^0 64/5 SH-SY5Y cells and ρ^0 A431 cells were produced and maintained as described (Miller *et al.*, 1996).

DNA isolation from cultured cells. Total cellular DNA was isolated from human cell lines by first removing the cells from the tissue culture flasks by treatment with 0.5 mM EDTA in Dulbecco's PBS. Cells were then pelleted by centrifugation at 2000*g* for 10 min, and DNA was extracted with DNAzol reagent (Molecular Research Center, Inc., Cincinnati, OH). Alternatively, cells were resuspended in water and lysed by placing into a 100°C water bath for 10 min. After cooling to room temperature, cellular debris was removed by centrifugation at 14,000*g* for 2 min. DNA concentrations were determined by UV absorption at 260 nm.

Isolation of mitochondria from brain tissue. Mitochondria were purified from human brain tissue, and mtDNA was extracted as described by Mecocci *et al.* (1994). Human parietal cortex tissue was homogenized in an isotonic solution (0.05 M Tris–HCl, pH 8.0, 0.21 M mannitol, 0.07 M sucrose, 3 mM calcium chloride) using a glass dounce homogenizer and a round bottom glass rod with 0.15-μm clearance. EDTA concentration was brought to 0.02 M, and the homogenate was centrifuged at 1500*g* for 20 min at 4°C. The supernatant was transferred to a new tube, centrifuged again at 1500*g* for 20 min at 4°C, transferred to a new tube, and centrifuged at 17,000*g* for 20 min at 4°C. The pellet consisting of mitochondria was resuspended in 40 mM Tris–HCl, pH 8.0, 10 mM magnesium chloride, containing DNase I and RNase A (Boehringer Mannheim, Indianapolis, IN) at concentrations of 40 units/ml and 0.02 mg/ml, respectively, and incubated for 45 min at 37°C. The organelle suspension was diluted to 20 ml with isotonic dilution buffer (0.05 M Tris–HCl, pH 8.0, 0.21 M mannitol, 0.07 M sucrose, 0.01 M EDTA) and centrifuged at 17,000*g* for 20 min at 20°C. The pellet was resuspended in isotonic dilution buffer, combined with an equal volume of lysis buffer (0.01 M Tris–HCl, pH 8.0, 0.1 M NaCl, 1 mM EDTA, 0.5% SDS) containing 400 μg/ml Proteinase K (Boehringer Mannheim), and incubated for 3 h at 37°C. DNA was purified by three phenol extractions followed by three chloroform:isoamyl alcohol (24:1) extractions and ethanol precipitation. The DNA was resuspended in buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA), and its concentration was determined by UV absorption at 260 nm.

Southern blot analysis. Total DNA isolated from white blood cells with DNAzol reagent, DNA isolated from ρ^0 118/5 SH-SY5Y cells with DNAzol reagent, and DNA extracted from purified human brain mitochondria were treated with *Nae*I and *Sac*I restriction endonucleases (New England Biolabs, Beverly, MA), separated by horizontal agarose gel electrophoresis, and blotted onto Nytran Plus membranes (Schleicher & Schuell, Keene, NH). Membranes were probed with an ~200-bp fragment of the ATP synthase subunit 8 (ATPase 8) gene that had been generated by PCR amplification of a DNA preparation from white blood cells (L-strand primer, 5′-TAGCATTAACCTTTTAAGTTAAAGA; H-strand primer, 5′-TCGTTCATTTTGGTTCTCA). The probe was labeled with [^{32}P]dATP (Amersham, Arlington Heights, IL) and the random priming method (Prime-a-Gene Labeling System, Promega). The blot was incubated with approximately 50 million cpm (5–10 ng) of probe for 16 h at 42°C in hybridization solution (6× SSC, 50% formamide, 1× Denhardt's solution, 1% SDS, and 100 μg/ml salmon sperm DNA). Blots were washed twice for 5 min in 6× SSC, 0.1% SDS at room temperature, once for 30 min in 2× SSC, 0.1% SDS at 42°C, and once for 30 min in 0.2× SSC, 0.1% SDS at 42°C, exposed to XAR 5 film (Kodak, Rochester, NY) for 1 to 4 days, and developed.

mRNA analysis. Total RNA was extracted with Trizol reagent (Gibco BRL) from buffy coats that had been prepared by the Accuspin procedure (Sigma). First-strand cDNA was synthesized with the SuperScript Preamplification System (Gibco BRL) using oligo(dT) primer. Subsequent PCR was performed with primers complementary to wildtype COII mtDNA sequence (Fahy *et al.*, 1997) and AmpliTaq DNA polymerase using standard PCR conditions and a Perkin–Elmer 9600 thermal cycler. Amplification products were purified and analyzed by the primer extension assay (Fahy *et al.*, 1997).

Affinity purification of mitochondria. Human cells grown in culture were harvested by scraping in MSB (0.21 M mannitol, 0.07 M sucrose, 0.05 M Tris–HCl, 0.01 M EDTA, pH 7.4; 10^7 cells/ml) and lysed by three freeze–thaw cycles. Cellular debris was removed by centrifugation at 1000*g* for 5 min. The mitochondria-enriched supernatants were used for subsequent immunoprecipitation steps. Monoclonal anti-mitochondrial antibody (MAB1273; Chemicon International Inc., Temecula, CA) was added to the mitochondria-enriched fractions for 2 h. Antibody–mitochondria complexes were isolated using a second antibody bound to magnetic beads (Dynal Inc., Great Neck, NY) according to the manufacturer's instructions. After extensive washing of the bead–antibody–mitochondria complexes with PBS/0.1% BSA, mtDNA was extracted from the complexes using DNAzol reagent.

Quantitative analysis of the numtDNA/mtDNA ratios. Amplification of cellular DNA isolated from platelet/white blood cell fractions and from cultured cells was performed according to the conditions described in Fahy *et al.* (1997) using PCR primers complementary to COI and COII mtDNA sequences. The resulting PCR products were purified and then used as templates in a competitive primer extension assay. *UlTma* DNA polymerase (Perkin–Elmer) was used to differentially elongate 5′-FAM-labeled primers by one or two nucleotides depending on the sequence of the template and the dNTP–ddNTP mixtures. DNA sequences at nucleotide positions 6366, 6483, 7146, 7650, and 7868 were interrogated, and the ratios of numtDNA and wildtype mtDNA sequences were determined based on the fluorescence intensities of the primer extension products (Fahy *et al.*, 1997).

Radiation hybrid mapping. Radiation hybrid (RH) screening by PCR was accomplished on the Stanford G3 panel (Research Genetics, Huntsville, AL). PCR primers were designed to select for the numtDNA sequence and contain nucleotide changes at positions 9325, 9329, 9335, 9540, 9545, and 9548 relative to the CRS (L-strand primer, 5′-TCCACTCCACAACCCTCCTT; H-strand primer, 5′-GGGCCAGTGTCCCCTAG). Human and hamster genomic DNA (Research Genetics) was used to confirm specific amplification of a 223-bp fragment (numtDNA marker) using AmpliTaq DNA polymerase (Perkin–Elmer) and standard PCR conditions. Analysis was performed on the RH Server operated by the Stanford Human Genome Center (SHGC; http://www-shgc.stanford.edu). The numtDNA marker was linked to more than 5000 framework markers and ESTs that have been characterized by the SHGC and Généthon.

RESULTS

Identification of numtDNA Sequence in a BAC Library

A screening strategy was devised to identify and isolate potential mtDNA-like sequences in nuclear DNA using a bacterial artificial chromosome library (Genome Systems, Inc.) that had been prepared from human leukocyte DNA. The average insertion length of this library (50–240 kb) should be sufficiently large to exclude true 16.5-kb mtDNA sequences. A PCR amplification reaction was targeted toward sequences that carry the nucleotide substitutions in the COI gene previously identified (Davis *et al.*, 1997; Wallace *et al.*, 1997) by using a primer pair that contained single nucleotide substitutions at the 3′-ends corresponding to mtDNA nucleotide positions G6366A and A7146G. The PCR screen of the library identified a single clone that was analyzed by sequencing of a portion of the insert (Fig. 1). This clone contains a 5840-bp contiguous DNA sequence with 98% identity to the CRS (Anderson *et al.*, 1981).

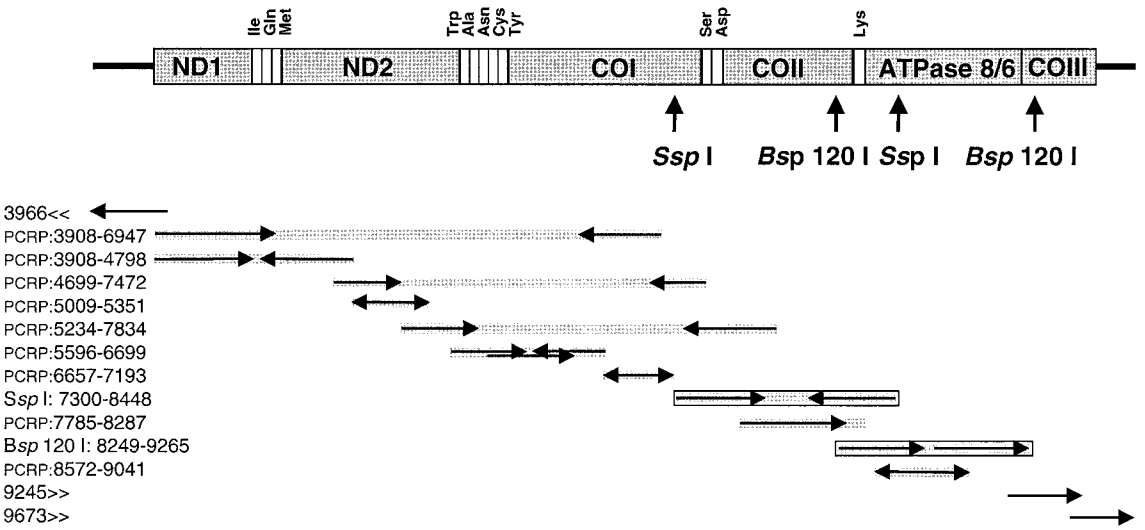


FIG. 1. Sequencing strategy of the numtDNA and adjacent sequences contained in the BAC clone. A schematic drawing of the numtDNA containing gene sequences for ND1, ND2, COI, COII, ATPase 8 and 6, and COIII (gray boxes), interspersed tRNA genes (white boxes), and adjacent chromosomal sequences (black bars) is shown. Restriction sites in the numtDNA for *Ssp*I at nucleotide positions 7300 and 8448 and for *Bsp*120I at nucleotide positions 8249 and 9265 are indicated by vertical arrows. DNA sequence data for the BAC clone insertion were obtained by direct sequencing of PCR products (PCRP; nucleotide positions were as indicated; gray bars) that were amplified from portions of the numtDNA sequence using primers complementary to the CRS (Anderson *et al.*, 1981). The purified PCR products were sequenced with primers located at the ends or at internal sites (horizontal black arrows inside gray bars). An *Ssp*I restriction fragment of 1.1 kb in length and a *Bsp*120I restriction fragment of 1.0 kb in length were subcloned and sequenced (*Ssp*I, *Bsp*120I; nucleotide numbers were as indicated) (boxed gray bars and horizontal black arrows). Both ends of the numtDNA sequence and adjacent chromosomal sequences were determined by sequencing the BAC clonal DNA using primers complementary to the CRS at nucleotide positions 3966, 9245, and 9673 (horizontal black arrows alone).

The region of sequence similarity of the BAC clone insertion with the human mtDNA spans nucleotide positions 3914 to 9755 relative to the CRS (Table 1). This putative numtDNA fragment is flanked by non-mitochondrial DNA sequences that lack significant sequence identity to any known nuclear DNA sequences accessible through GenBank and EMBL database searches. Unlike many previously identified numtDNA sequences, this sequence is largely in-frame relative to the human mitochondrial genome across the entire sequence, with the exception of a 2-bp deletion at nucleotide positions 8196 and 8197 (Wallace *et al.*, 1997). Earlier investigators had analyzed two small portions of this numtDNA that contained the COI and COII gene sequences (Wallace *et al.*, 1997; Hirano *et al.*, 1997). The 5840-bp sequence contains a truncated NADH dehydrogenase subunit 1 (ND1; NMND1P1*) gene, complete NADH dehydogenase subunit 2 (ND2; NMND2P1*), and cytochrome *c* oxidase subunit 1 (COI; NMCOIP2*) gene, a cytochrome *c* oxidase subunit 2 (COII; NMCOIIP1) gene with the 2-bp deletion, complete ATP synthase subunits 8 and 6 (ATPase 8 and 6; NMATP8P1*, NMATP6P1*) genes, a truncated cytochrome *c* oxidase subunit 3 (COIII; NMCOIIIP1*) gene, and full-length sequences for 11 interspersed tRNAs.

Nucleotide substitutions were found in all seven structural genes of the 5840 bp of the numtDNA sequence (Table 1) compared to the CRS (Anderson *et al.*, 1981), some of which would be synonymous substitutions while others would constitute nonsynonymous substitutions if translated by the mitochondrial genetic code. In addition to the previously reported nonsynonymous substitutions in COI and COII (Davis *et al.*, 1997; Wallace *et al.*, 1997), nonsynonymous substitutions also occur at nucleotide positions 4048, 5041, 5320, 8545, 8677, 8701, 9325, and 9329. Nucleotide differences at positions 4769 and 8860 appear to result from rare polymorphisms in the CRS mtDNA, and differences at positions 4985 and 9559 are errors that were reported in the original CRS (Neil Howell, Galveston, TX, February 1999, pers. comm.). Two nucleotide substitutions were found in $tRNA^{Ile}$ (NMTTIP1*), three in $tRNA^{Gln}$ (NMTTQP1*), one each in $tRNA^{Met}$ (NMTTMP1*) and $tRNA^{Cys}$ (NMTTCP1*) and, as previously reported (Wallace *et al.*, 1997), one each in $tRNA^{Tyr}$ and $tRNA^{Asp}$. No substitutions were present in $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Ser(UCN)}$, and $tRNA^{Lys}$. A 2-bp deletion at nucleotide positions 8196 and 8197 is consistent with previously reported deletions at positions 8197 and 8998 (Wallace *et al.*, 1997).

There are 87 nucleotide substitutions relative to human mtDNA within the entire 5840-bp numtDNA sequence. Among the 78 nucleotide changes in the coding regions, 56 (71.8%) are synonymous substitutions, whereas 13 (16.7%) would lead to amino acid changes if translated by the mitochondrial genetic code. Nine additional base substitutions are in tRNA sequences. Eighty-three (95.4%) of all these substitutions are transitions, and four are transversions (4.6%). The high bias toward transitions over transversions is characteristic of the mutational patterns in mitochon-

TABLE 1

Comparison of mtDNA and numtDNA Sequences

Gene	Nucleotide position	Human mtDNA	Human numtDNA	Gene	Nucleotide position	Human mtDNA	Human numtDNA
ND1	4048	G	A	COI	7146	A	G
	4104	A	G		7232	C	T
$tRNA^{Ile}$	4312	C	T		7256	C	T
	4318	C	T		7316	G	A
$tRNA^{Gln}$	4375	C	G	$tRNA^{Asp}$	7521	G	A
	4382	C	A	COII	7650	C	T
	4398	C	T		7705	T	C
$tRNA^{Met}$	4456	C	T		7810	C	T
ND2	4496	C	T		7868	C	T
	4736	T	C		7891	C	T
	4769	A	G		7912	G	A
	4856	T	C		8021	A	G
	4904	C	T		8065	G	A
	4914	C	T		8140	C	T
	4940	C	T		8152	G	A
	4958	A	G		8167	T	C
	4985	G	A		8196–8197	AC	deletion
	4991	G	A		8203	C	T
	5041	T	C	ATPase8	8392	G	A
	5147	G	A		8455	C	T
	5320	C	T		8461	C	T
	5351	A	G		8503	T	C
	5387	C	T		8545	G	A
	5426	T	C	ATPase6	8545	G	A
	5471	G	A		8655	C	T
	5474	A	G		8677	A	C
	5498	A	G		8701	A	G
$tRNA^{Cys}$	5821	G	A		8718	A	G
$tRNA^{Tyr}$	5840	C	T		8860	A	G
COI	6023	G	A		8943	C	T
	6221	T	C		9060	C	A
	6242	C	T		9075	C	T
	6266	A	C		9103	C	T
	6299	A	G		9168	C	T
	6366	G	A		9175	C	T
	6383	G	A	COIII	9254	A	G
	6410	C	T		9325	T	C
	6452	C	T		9329	G	C
	6483	C	T		9335	C	T
	6512	T	C		9434	A	G
	6542	C	T		9540	T	C
	6569	C	A		9545	A	G
	6641	T	C		9548	G	A
	6935	C	T		9559	G	C
	6938	C	T		9629	A	G

Note. The numtDNA sequence was compared to nucleotide position 3914 to 9755 of modern human mtDNA (Anderson *et al.*, 1981) by sequence alignment using the Jotun Hein Method (DNASTAR software program, DNASTAR Inc., Madison, WI). Differences between mtDNA and numtDNA are listed.

drial DNA, but not that of nuclear DNA (Brown *et al.*, 1982). This pattern suggests that this numtDNA molecule may represent mtDNA from a hominid ancestor that was translocated and fixed in the nuclear genome during evolution.

Copy Numbers of the 5.8-kb numtDNA and mtDNA in Cellular DNA Preparations

The nucleotide substitution A → C at position 6266 in the COI gene of the numtDNA sequence created a 6-bp *Nae*I restriction recognition site that is not present in human wildtype mtDNA. We used this difference to distinguish mtDNA from the numtDNA sequence by Southern blot analysis of human cellular DNA isolated from white blood cells (Fig. 2a). DNA from two normal control individuals was restricted with *Sac*I, which cleaved at nucleotide position 9647 in the COII gene of both the mtDNA and the numtDNA, and with *Nae*I, which cleaved at nucleotide position 932 in the 12S rRNA gene of mtDNA and nucleotide position 6266 in the nmtDNA. A wildtype mitochondrial ATPase 8 probe detected the resulting 8715-bp mtDNA and 3381-bp numtDNA fragments. The signal intensities of the two DNA fragments indicated their

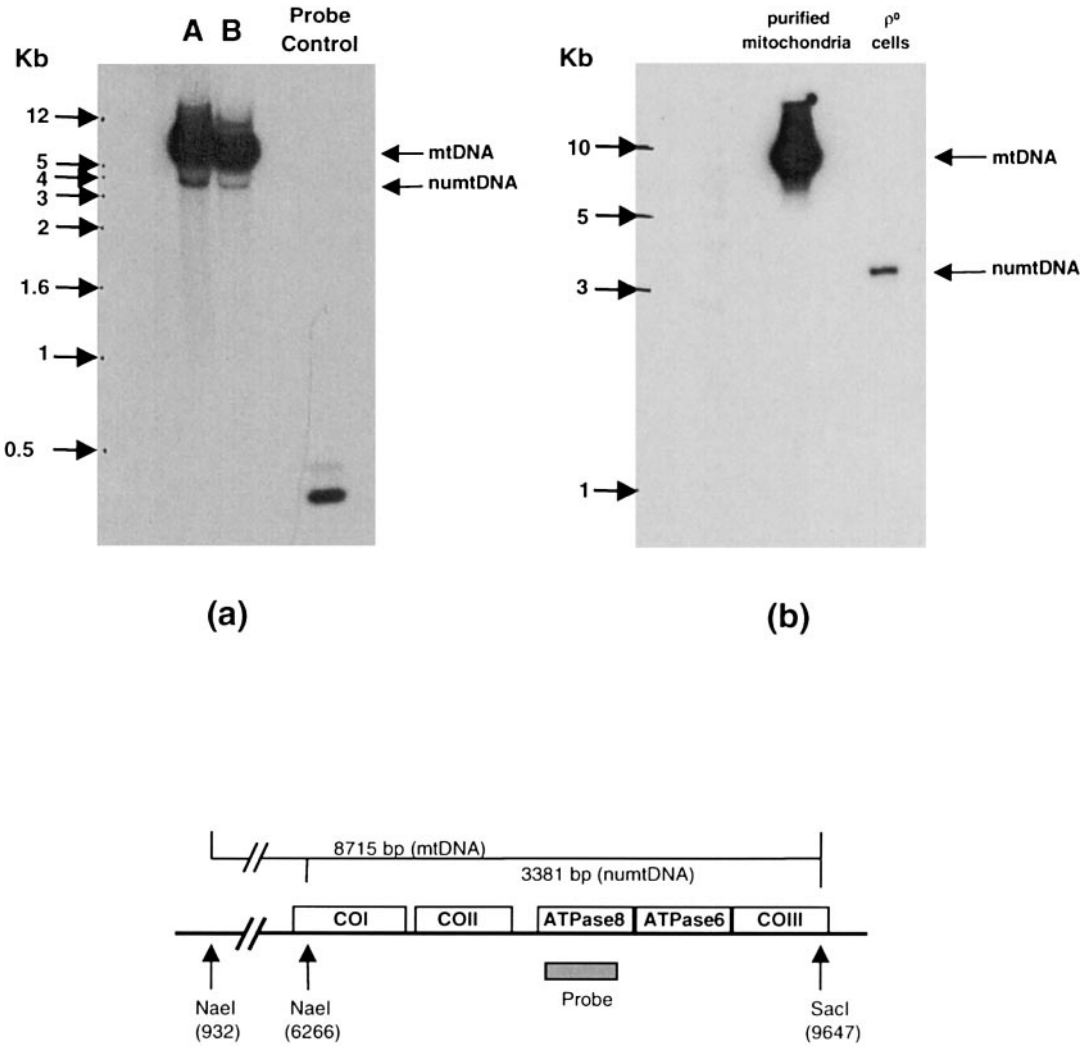


FIG. 2. Southern blot analysis of numtDNA and mtDNA sequences in white blood cells, ρ^0 SH-SY5Y cells and purified human brain mitochondria. (**a**) Total DNA was isolated with DNAzol reagent from white blood cells of two control individuals (**A, B**), and (**b**) DNA was isolated from ρ^0 SH-SY5Y cells with DNAzol reagent and from purified human brain mitochondria with Proteinase K digestion. Approximately 10 μg of each DNA sample was restricted with *Nae*I and *Sac*I and probed with a portion of the wildtype ATPase 8 gene sequence. Wildtype mtDNA carries a *Nae*I site at nucleotide position 932 that yields a 8715-bp restriction fragment in combination with *Sac*I restriction at nucleotide position 9647. *Nae*I recognizes a restriction site present in the numtDNA sequence, which is created by the nucleotide substitution at position 6266 and is not present in the wildtype mtDNA. By cleavage of the numtDNA at this site and at nucleotide position 9647 by *Sac*I, a 3381-bp restriction fragment is created. An unlabeled aliquot (~4 ng) of the 200-bp ATPase 8 probe ((**a**): probe control) shows appropriate hybridization signal. Exposure times of the blots to X-ray film were (**a**) 24 h and (**b**) 4 days.

relative abundance in the cellular DNA preparation, suggesting a low copy number or possibly a single copy of this numtDNA within the genomic DNA.

The numtDNA Molecule Is Not Expressed as a Mitochondrially Encoded Gene

Because structural mitochondrial genes are transcribed and processed into individual poly(A) mRNAs, reverse transcription followed by PCR amplification can be used as a direct method that can distinguish an expressed mitochondrial gene from a nuclear pseudogene (Collura *et al.*, 1996). RNA was isolated from white blood cells, and RT-PCR was used to amplify a putative COII messenger RNA encoded by the numtDNA sequence.

Simultaneous detection and quantification of the nearly identical numtDNA and wildtype mtDNA amplicons can be accomplished using a competitive primer extension assay (Fahy *et al.*, 1997). Any of the divergent nucleotides between the numtDNA and the mtDNA molecule should be equally useful for identification and quantification purposes. A fluorophor-labeled primer, designed to anneal to both the numtDNA and the mtDNA amplicons just upstream of the nucleotide to be interrogated, a thermostable, high-fidelity DNA polymerase, and a selected mixture of deoxynucleotides and dideoxynucleotides are used in minisequencing reactions. The differentially extended fluorescent primer products are separated by gel electrophoresis and distinguished by their size (Fig. 3). The relative amounts of the numtDNA versus the mtDNA sequences are estimated from the fluorescence intensi-

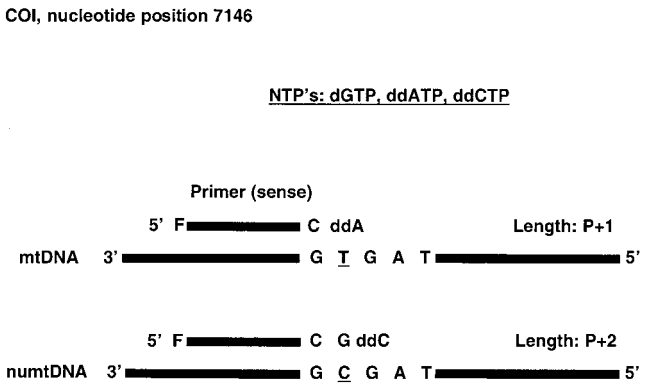


FIG. 3. Analysis of the DNA sequence at nucleotide position 7146 of the COI gene in mtDNA and numtDNA by the primer extension assay. Interrogation of the DNA sequence at nucleotide position 7146 is presented as an example of the primer extension method described in detail elsewhere (Fahy *et al.*, 1997). The DNA sequence at position 7146 consists of an A (mtDNA) or a G (numtDNA) in the sense strand corresponding to a T or a C, respectively, in the antisense strand (underlined). *UlTma* DNA polymerase was used with a nucleotide combination of dGTP, ddATP, and ddCTP to extend a fluorescein (F) 5′-labeled sense primer by either one (mtDNA amplification product template) or two (numtDNA amplification product template) nucleotides. The extended primer products were distinguished electrophoretically by their size (P + 1, P + 2) and relative fluorescent amounts were determined by the GENESCAN software program (Applied Biosystems Perkin–Elmer). Quantitative analysis was carried out using a standard curve generated from known ratios of plasmids containing mtDNA and numtDNA inserts.

ties associated with electrophoretically separated primer extension products and are calculated as (numtDNA/(mtDNA + numtDNA)) × 100. This assay is linear and reliably detects between 1 and 3% of a nucleotide substitution in a background of wildtype sequence (Fahy *et al.*, 1997; Ghosh *et al.*, 1996). Values below 1% reflect the nonspecific background noise inherent in this assay and define the limit of sensitivity of the method.

DNA obtained from RT-PCR amplification was interrogated for nucleotide positions 7650 and 7868 by the primer extension assay (Fahy *et al.*, 1997). No sequence identical to the COII portion of the numtDNA was detectable in human leukocytes, whereas mRNA for the human wildtype COII genes was detectable (Fig. 4). This finding suggests that this numtDNA sequence is not expressed as functional mitochondrial mRNA.

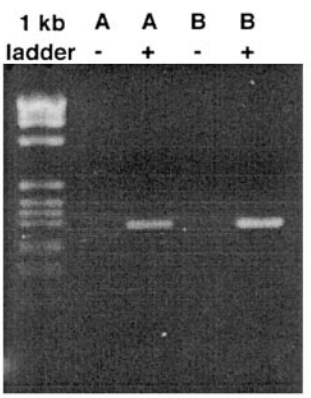
The 5.8-kb numtDNA Sequence Is Present in ρ^0 Cell Lines

To provide additional support for a nuclear origin of the 5.8-kb DNA sequence, human cell lines depleted of mtDNA (ρ^0 cells) were analyzed for the presence of this sequence. In a Southern blot experiment, DNA isolated from ρ^0 SH-SY5Y neuroblastoma cells was restricted with *Sac*I and *Nae*I and hybridized with a wildtype mitochondrial ATPase 8 probe, which detected the resulting 3381-bp numtDNA fragment (Fig. 2b).

A different experimental approach was used when the numtDNA sequence was amplified using PCR primers internal to the numtDNA sequence. These primers annealed to identical sequences in both mtDNA and numtDNA, and they were used to amplify DNA from two human cell lines (SH-SY5Y neuroblastoma cells, A431 carcinoma cells) depleted of mtDNA (ρ^0). If the putative numtDNA sequence were actually of mitochondrial origin, it should not be present in these ρ^0 cell lines. Treatment of SH-SY5Y cells with varying amounts of ethidium bromide leads to a concentration-dependent decrease in mtDNA content (Miller *et al.*, 1996). With decreasing concentrations of mtDNA, the ratio of the numtDNA molecule to the sum of multicopy wildtype human mtDNA plus numtDNA should approach unity as the concentration of mtDNA approaches zero. Two DNA isolation procedures and their abilities to recover mtDNA versus nuclear DNA were compared.

The nucleotide position relative to 7146 in mtDNA was interrogated by the primer extension assay (Fig. 3) (Fahy *et al.*, 1997). Given the limit of sensitivity of the assay (see above), the low-copy-number numtDNA sequence should be undetectable in SH-SY5Y cells that contain abundant copies of mtDNA. Indeed, SH-SY5Y cell lines treated with 1.0 μg/ml or less of ethidium bromide for 64 days were shown to retain high levels of

a



b

Patient #	% numtDNA at two nucleotide positions	
	NP 7650	NP 7868
416 (A)	0.4	0.0
1516 (B)	0.7	0.0

FIG. 4. Analysis of RNA transcripts of COII sequences in white blood cells. (**a**) RNA was isolated from white blood cells of two control individuals (**A, B**) and subjected to RT-PCR. Aliquots of the completed reactions were analyzed by horizontal agarose gel electrophoresis. Control reactions containing no reverse transcriptase are indicated by (−) and showed no RT-PCR product; reactions carried out in the presence of reverse transcriptase (+) yielded products of expected size. (**b**) RT-PCR products were analyzed by competitive primer extension assay (Fahy *et al.*, 1997) for nucleotide positions 7650 and 7868. The fraction of numtDNA sequences was calculated as (numtDNA/(mtDNA + numtDNA)) × 100.

TABLE 2

Relative Amounts of numtDNA and mtDNA in Cells Treated with Various Concentrations of Ethidium Bromide

Cell type	μg/ml EtBr	Days of treatment	numtDNA (%)[a]	numtDNA (%)[b]
A431	0.0	73	1.8	1.3
A431	0.1	73	100.0	100.0
A431	0.25	73	100.0	100.0
SY5Y	0.0	64	0.0	0.0
SY5Y	0.5	64	0.0	2.6
SY5Y	1.0	64	0.0	3.4
SY5Y	5.0	64	32.1	80.5
SY5Y	5.0	118	100.0	100.0

Note. SH-SY5Y and A431 cells were treated with various amounts of ethidium bromide for defined periods of time, and cellular DNA was isolated using a DNAzol and a boiling procedure. The DNA was subjected to PCR, and the PCR products were interrogated at nucleotide position 7146 by the primer extension assay (Fahy *et al.*, 1997). The fraction of numtDNA sequences was calculated as (numtDNA/(mtDNA + numtDNA)) × 100.

[a] DNAzol DNA isolation procedure.

[b] Boiling DNA isolation procedure.

mtDNA (Table 2). Conversely, extremely small numbers of mtDNA molecules that may have been present in a ρ^0 cell population were below the level of detection by this primer extension assay. This condition was exemplified by cells treated with 5.0 μg/ml of ethidium bromide for 64 and 118 days that contained few or no detectable mtDNA copies, respectively. These results suggested that the numtDNA sequence was present in nonmitochondrial compartments of SH-SY5Y cells. Furthermore, A431 cells were more susceptible to ethidium bromide treatment than SH-SY5Y cells, and they contained no detectable mtDNA after 73 days of treatment with 0.1 μg/ml or less of ethidium bromide. An interesting observation was the difference in recovery of mtDNA versus nuclear DNA in the two DNA isolation procedures. The DNAzol procedure appeared to recover more mtDNA (or less nuclear DNA) while the boiling procedure generally yielded more nuclear DNA (or less mtDNA).

Purified Mitochondria Lack the 5.8-kb numtDNA Sequence

To confirm the above data that suggest that the numtDNA sequence does not reside within mitochondria, we purified mitochondria from a human parietal cortex brain sample and extracted DNA from the purified mitochondria fraction. The mtDNA was digested with *Sac*I and *Nae*I and subjected to Southern blot hybridization with a wildtype mitochondrial ATPase 8 probe, which detected a 8715-bp mtDNA fragment only and no 3381-bp numtDNA fragment (Fig. 2b).

Furthermore, we extracted and analyzed DNA from immunopurified mitochondria. Antibodies that recognize epitopes on the outer surface of the mitochondria can be used to immunopurify mitochondria from other cellular components. Absence of the numtDNA molecule in immunopurified mitochondria would provide additional evidence for an extramitochondrial locus for this DNA sequence.

Mitochondria were isolated from SH-SY5Y ρ^0 cells treated with 5 μg/ml of ethidium bromide for 118 days and from A431 ρ^0 cells treated with 0.25 μg/ml of ethidium bromide for 73 days, as well as untreated SH-SY5Y and A431 cells, using the antibody MAB1273. Following cellular lysis, mitochondria were immunopurified from SH-SY5Y cells, ρ^0 SH-SY5Y cells, A431 cells, and ρ^0 A431 cells, and DNA was extracted. The competitive primer extension assay was utilized as described above to interrogate nucleotide positions 6366, 6483, 7650, and 7868 (Fahy *et al.*, 1997) and to quantify relative amounts of numtDNA and mtDNA calculated as (numtDNA/(mtDNA + numtDNA)) × 100. These assays revealed that the DNA isolated from SH-SY5Y and A431 mitochondria contained only mtDNA (Table 3) and no detectable numtDNA above the assay's limit of detection. Interestingly, PCR of the ρ^0 SH-SY5Y mitochondria sample provided an amplification product, suggesting that there still were a few copies of residual mtDNA in the ρ^0 SH-SY5Y cell population. This finding was corroborated by primer extension analysis of the amplification product that revealed the sequence to be of mtDNA

TABLE 3

Relative Amounts of numtDNA and mtDNA in Immunopurified Mitochondria from Untreated Cells and Ethidium Bromide-Treated ρ^0 Cells

Cell type	μg/ml EtBr	Days of treatment	% numtDNA at four nucleotide positions			
			NP 6366	NP 6483	NP 7650	NP 7868
SH-SY5Y	0.0	64	0.0	0.5	0.0	0.1
SH-SY5Y ρ^0	5	118	0.0	0.0	0.0	0.3
A431	0.0	73	0.0	0.6	0.2	0.1
A431 ρ^0	0.25	73	No amplified DNA	No amplified DNA	No amplified DNA	No amplified DNA

Note. Mitochondria were isolated from SH-SY5Y and A431 cells and from the same cell lines but following extensive treatment with ethidium bromide, using an antibody raised against mitochondrial surface protein. DNA was extracted and subjected to PCR. The PCR products were analyzed by competitive primer extension assay (Fahy *et al.*, 1997) for nucleotide positions 6366, 6483, 7650, and 7868. The percentage of numtDNA sequences was calculated as (numtDNA/(mtDNA + numtDNA)) × 100.

origin and the absence of numtDNA above the assay's limit of detection. No DNA could be amplified from immunopurified mitochondria from the ρ^0 A431 cell line, thus demonstrating the absence of both the mtDNA and the numtDNA sequence from the immunopurified mitochondria.

Chromosomal Localization of the numtDNA Sequence

Localization of the numtDNA sequence within the nuclear DNA was accomplished by radiation hybrid (RH) mapping (Boehnke *et al.*, 1991; Walter *et al.*, 1994). The Stanford G3 RH panel consists of more than 80 whole-genome radiation hamster–human hybrids and 14,000 characterized chromosomal markers (available at the SHGC). Eighty-three hybrid clones of the G3 RH panel and both parental human and hamster control cells were screened with PCR primers homologous to a portion of the COIII gene region within the numtDNA sequence. An amplification product of the expected 223-bp size was obtained from hybrid clones 3, 11, 33, 40, 49, 50, 59, 64, 73, and 75, as well as from the human control cells and not from the hamster control cells. The closest SHGC markers identified were SHGC-57364 with positive hybrid clones 3, 11, 33, 40, 49, 50, 59, 64, 73, and 75, SHGC-4723 with positive hybrid clones 11, 33, 49, 50, 51, 59, 64, 68, 73, and 75, and SHGC-428 with positive hybrid clones 9, 11, 40, 49, 50, 51, 59, 64, 68, 73, and 75. All three markers were localized to a very distal portion of chromosome 1 at distances to the numtDNA of 0, 26, and 32 cR, which is roughly equivalent to 0, 676, and 832 kb (Fig. 5). SHGC-4723 and SHGC-428 are ordered markers linked to Bin 1 of chromosome 1 at GDB Locus D1S2565 and D1S243, respectively. SHGC-57364 is a positioned marker, also linked to Bin 1 of chromosome 1, that has been oriented at a 26-cR distance of SHGC-4723 (LOD 7.11). Interestingly, this marker (GenBank Accession No. T10998) originated from a 230-nucleotide sequence with partial similarity to the mitochondrial gene for cytochrome *c* oxidase subunit 2. It is conceivable that the PCR primers used for identifying this marker in the clones where it was found may also have amplified the numtDNA sequence. However, independent confirmation of the numtDNA colocalization is provided by the specific COIII primer set. These data demonstrated unambiguously the nuclear chromosomal location of this numtDNA and its presence as a single-copy-number sequence.

Evolutionary Divergence of the numtDNA Sequence and Primate mtDNA

Previously, we and others reported that a portion of the numtDNA molecule, encoding COI and COII sequences, has features in common with the mtDNA sequences of the modern chimpanzee, gorilla, and orangutan (Davis *et al.*, 1997; Wallace *et al.*, 1997). Alignment of the full numtDNA sequence with the mtDNA sequence of human and other primates revealed similar levels of overall sequence identity (Table 4). The sequence divergence between the numtDNA sequence and the human wildtype mtDNA sequence was 1.9% and varied between 0.6 and 2.4% across the regions homologous to the mitochondrial protein encoding genes. The overall sequence divergence between human and chimpanzee mtDNAs was 9.7%, between human and gorilla mtDNAs was 12.2%, and between human and orangutan mtDNAs was 17.9%. The high degree of homology of the numtDNA sequence with modern human mitochondrial DNA suggests a recent translocation of the numtDNA sequence to the nucleus during hominid evolution.

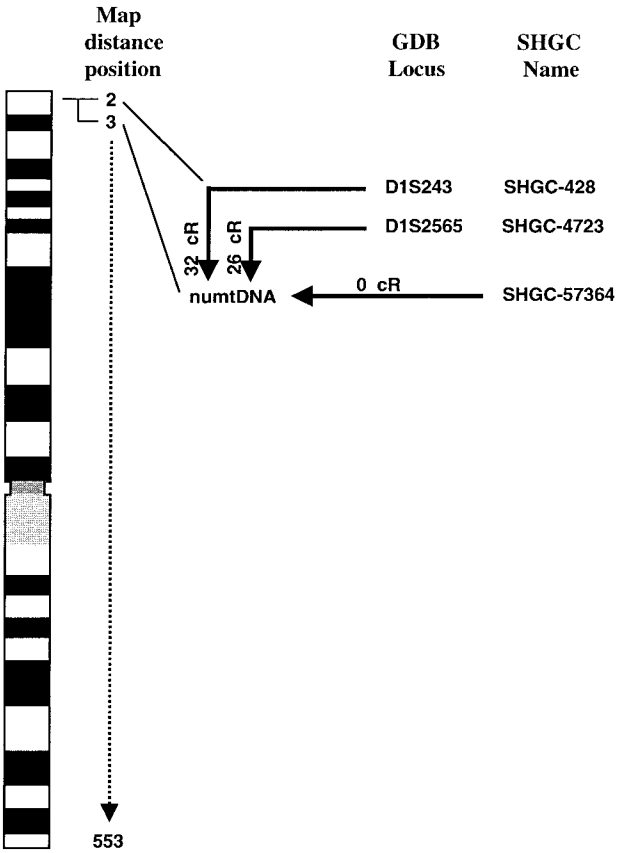


FIG. 5. Localization of the numtDNA sequence on chromosome 1. The Stanford G3 radiation hybrid panel was used to screen the numtDNA sequence against more than 5000 markers available at the Stanford Human Genome Center. Linkage of the numtDNA was established with three SHGC markers localized to a very distal portion of chromosome 1 at GDB locis D1S243 and D1S2565. The distance of the numtDNA to the three markers is indicated in centirays (1 cR corresponds roughly to 26 kb).

DISCUSSION

The 5840-bp mtDNA-like nuclear sequence that we report here is unusual in its length, continuity of sequence (ND1-COIII), pattern of base changes, and paucity of frameshift deletions, insertions, or stop codons (only one 2-bp deletion within this entire sequence). Most nuclear mtDNA-like sequences have been reported as short fragments that lack contiguity and

TABLE 4

Similarity of Human mtDNA with Human numtDNA and mtDNAs from Modern Hominids

	Percentage similarity with human wildtype mtDNA			
	Human numtDNA	Chimpanzee	Gorilla	Orangutan
ND1	99.4	89.7	88.9	84.1
ND2	98.0	90.0	87.0	81.0
COI	98.0	91.0	89.0	85.0
COII	98.0	90.0	87.0	85.0
ATPase 8	97.6	91.3	87.0	74.9
ATPase 6	98.0	90.0	88.0	80.0
COIII	98.0	90.0	88.0	85.0
Average similarity	98.1	90.3	87.8	82.1

Note. Human mtDNA sequence was compared to the human 5.8-kb numtDNA and the mtDNAs from the great apes by sequence alignment using the Jotun Hein Method (DNASTAR software program, DNASTAR Inc.).

contain termination codons, frameshift deletions, and mutations (Fukuda *et al.*, 1985; Hu and Thilly, 1994, 1995; Kamimura *et al.*, 1989; Shay and Werbin, 1992; Zischler *et al.*, 1995). This numtDNA sequence would encode intact proteins and produce functional tRNA molecules if it were located in the mitochondria and not in the nucleus.

We previously reported a mtDNA-like sequence that was a fragment of this numtDNA molecule. This numtDNA sequence was present in the DNA extracted from the blood of most human donors. The presence of Alzheimer disease (AD) was reported to be associated with an elevation of the relative content of this numtDNA fragment (Davis *et al.*, 1997). In the light of the findings described here, the elevation of the numtDNA content relative to the amount of mtDNA could reflect a decrease in the mtDNA, an increase in nuclear DNA, or both of these events, in DNA sample preparation using our Accuspin/heat lysis procedure. Hirano *et al.* (1997) provided evidence that less mtDNA was extracted from blood using heat for cell lysis. As indicated by the data presented in Table 2 (see entry for SH-SY5Y, 5 μg/ml EtBr), a similar finding was noted in cell lines when less mtDNA was recovered with a "boiling DNA isolation procedure" (80.5% numtDNA) compared to a "DNAzol DNA isolation procedure" (32.1% numtDNA). The phenomenon responsible for the significant elevation of the ratio of this numtDNA fragment relative to mtDNA in the blood of Alzheimer disease patients remains to be elucidated. Nevertheless, the previous findings remain robust and have been expanded to include 1046 AD cases and 376 control cases (S. S. Ghosh *et al.*, unpublished data).

Several studies (Davis and Parker, 1998; Hirano *et al.*, 1997; Wallace *et al.*, 1997) have proposed that a fragment of this DNA sequence might be located in the nucleus. However, those studies did not provide direct, conclusive evidence to support this contention because the complete numtDNA sequence and its nuclear flanking sequences were not identified. We have characterized this numtDNA sequence in its entirety and have shown conclusively that it is located in nuclear and not in mitochondrial DNA and that it is not expressed as mitochondrial mRNA. We have identified for the first time the nonmitochondrial DNA sequences that flank this numtDNA molecule, and we have localized this numtDNA to the very distal portion of chromosome 1. Additional confirmation of this distal chromosomal location was provided by the observation that our BAC clone cross reacted with several telomeric chromosomal sequences in a fluorescence *in situ* hybridization experiment (results not shown), indicating that this numtDNA sequence was located at the end of a chromosome and that our BAC clone contained telomeric sequences.

The contiguity of this numtDNA sequence with nonmitochondrial upstream and downstream flanking sequences, the lack of detectable mitochondrially encoded mRNA transcribed from this sequence, the presence of this DNA sequence in two cell lines that lack mtDNA and its absence in immunopurified mitochondria preparations all provide compelling evidence that this numtDNA sequence is contained only within nuclear DNA and that it is not a true mitochondrial DNA sequence. This numtDNA molecule contains phylogenetically ancient mitochondrial sequences that are largely identical to the mtDNA of modern humans, chimpanzee, gorilla, and orangutan. Based on the degree of sequence similarities of this numtDNA with modern human mtDNA and mtDNA of the great apes, it is estimated that this sequence was transferred to the nucleus during evolution long after the divergence of humans from other nonhuman primates (Wallace *et al.*, 1997).

The discovery of this numtDNA sequence and other similar recently translocated mtDNA sequences open new avenues for the study of mtDNA evolution. Furthermore, the striking similarity of the 5.8-kb fragment to modern human mtDNA highlights the necessity of exercising utmost care during the identification of mtDNA sequences that are associated with human mitochondrial disease. Thus, findings of "heteroplasmic" mtDNA mutations require critical evaluation to uncover errors that may arise through contamination with recently translocated, hitherto unidentified numtDNA sequences.

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REFERENCES

Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A.,

Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G. (1981). Sequence and organization of the human mitochondrial genome. *Nature* **290:** 456–465.

Arctander, P. (1995). Comparison of a mitochondrial gene and a corresponding nuclear pseudogene. *Proc. R. Soc. London B* **262:** 13–19.

Blanchard, J. L., and Schmidt, G. W. (1996). Mitochondrial DNA migration in yeast and humans: Integration by a common end-joining mechanism and alternative perspectives on nucleotide substitution patterns. *Mol. Biol. Evol.* **13:** 537–548.

Boehnke, M., Lange, K., and Cox, D. R. (1991). Statistical methods for multipoint radiation hybrid mapping. *Am. J. Hum. Genet.* **49:** 1174–1188.

Brown, W. M., Prager, E. M., Wang, A., and Wilson, A. C. (1982). Mitochondrial DNA sequences of primates: Tempo and mode of evolution. *J. Mol. Evol.* **18:** 225–239.

Collura, R. V., and Stewart, C. (1995). Insertions and duplications of mtDNA in the nuclear genomes of old world monkeys and hominoids. *Nature* **378:** 485–489.

Collura, R. V., Auerbach, M. R., and Stewart, C. (1996). A quick, direct method that can differentiate expressed mitochondrial genes from their nuclear pseudogenes. *Curr. Biol.* **6:** 1337–1339.

Corral, M., Baffet, G., Kitzis, A., Paris, B., Tichonicky, L., Kruh, J., Guguen-Guillouzo, C., and Defer, N. (1989). DNA sequences homologous to mitochondrial genes in nuclei from normal rat tissues and from rat hepatoma cells. *Biochem. Biophys. Res. Commun.* **162:** 258–264.

Davis, R. E., Miller, S., Herrnstadt, C., Ghosh, S. S., Fahy, E., Shinobu, L. A., Galasko, D., Thal, L. J., Beal, M. F., Howell, N., and Parker, W. D. (1997). Mutations in mitochondrial cytochrome *c* oxidase genes segregate with late-onset Alzheimer disease. *Proc. Natl. Acad. Sci. USA* **94:** 4526–4531.

Davis, J. N., and Parker, W. D. (1998). Evidence that two reports of mtDNA cytochrome c oxidase "mutations" in Alzheimer's disease are based on nDNA pseudogenes of recent evolutionary origin. *Biochem. Biophys. Res. Commun.* **244:** 877–883.

Fahy, E., Nazarbaghi, R., Zomorrodi, M., Herrnstadt, C., Parker, W. D., Davis, R. E., and Ghosh, S. S. (1997). Multiplex fluorescence-based primer extension method for quantitative mutation analysis of mitochondrial DNA and its diagnostic application for Alzheimer's disease. *Nucleic Acids Res.* **25:** 3102–3109.

Fukuda, M., Wakasugi, S., Tsuzuki, T., Nomiyama, H., and Shimada, K. (1985). Mitochondrial DNA-like sequences in the human nuclear genome. *J. Mol. Biol.* **186:** 257–266.

Ghosh, S. S., Fahy, E., Bodis-Wollner, I., Sherman, J., and Howell, N. (1996). Longitudinal study of a heteroplasmic 3460 Leber hereditary optic neuropathy family by multiplexed primer-extension analysis and nucleotide sequencing. *Am. J. Hum. Genet.* **58:** 325–334.

Hadler, H. I., Dimitrijev, B., and Mahalingam, R. (1983). Mitochondrial DNA and nuclear DNA from normal rat liver have a common sequence. *Proc. Natl. Acad. Sci. USA* **80:** 6495–6499.

Hirano, M., Shtilbans, A., Mayeux, R., Davidson, M. M., DiMauro, S., Knowles, J., and Schon, E. A. (1997). Apparent mtDNA heteroplasmy in Alzheimer's disease patients and in normals due to PCR amplification of nucleus-embedded mtDNA pseudogenes. *Proc. Natl. Acad. Sci. USA* **94:** 14894–14899.

Hu, G., and Thilly, W. G. (1994). Evolutionary trail of the mitochondrial genome as based on human 16S DNA pseudogenes. *Gene* **147:** 197–204.

Hu, G., and Thilly, W. G. (1995). Multi-copy nuclear pseudogenes of mitochondrial DNA reveal recent acute genetic changes in the human genome. *Curr. Genet.* **28:** 410–414.

Kamimura, N., Ishii, S., Liandong, M., and Shay, J. W. (1989). Three separate mitochondrial DNA sequences are contiguous in the human genomic DNA. *J. Mol. Biol.* **210:** 703–707.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X., and Wilson, A. C. (1989). Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. USA* **86:** 6196–6200.

Lee, L. G., Spurgeon, S. L., Heiner, C. R., Benson, S. C., Rosenblum, B. B., Menchen, S. M., Graham, R. J., Constantinescu, A., Updhya, K. G., and Cassel, J. M. (1997). New energy transfer dyes for DNA sequencing. *Nucleic Acids Res.* **25:** 2816–2822.

Lopez, J. V., Yuhki, N., Masuda, R., Modi, W., and O'Brien, S. J. (1994). Numt, a recent transfer and tandem amplification of mitochondrial DNA to the nuclear genome of the domestic cat. *J. Mol. Evol.* **39:** 174–190.

Louis, E. J., and Haber, J. E. (1991). Evolutionary recent transfer of a group I mitochondrial intron to telomere regions in *Saccharomyces cerevisiae*. *Curr. Genet.* **20:** 411–415.

Mecocci, P., MacGarvey, U., and Beal, M. F. (1994). Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. *Ann. Neurol.* **36:** 747–751.

Miller, S. M., Trimmer, P. A., Parker, W. D., and Davis, R. E. (1996). Creation and characterization of mitochondrial DNA-depleted cell lines with neuronal-like properties. *J. Neurochem.* **67:** 1897–1907.

Mullis, K. B., and Faloona, F. (1987). Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods Enzymol.* **155:** 335–350.

Nomiyama, H., Tsuzuki, T., Wakasugi, S., Fukuda, M., and Shimada, K. (1984). Interruption of a human nuclear sequence homologous to mitochondrial DNA by a member of the *Kpn*I 1.8 kb family. *Nucleic Acids Res.* **12:** 5225–5234.

Nomiyama, H., Fukuda, M., Wakasugi, S., Tsuzuki, T., Shimada, K., and Miyata, T. (1985). Molecular structures of mitochondrial-DNA-like sequences in human nuclear DNA. *Nucleic Acid Res.* **13:** 1649–1658.

Perna, N. T., and Kocher, T. D. (1996). Mitochondrial DNA: Molecular fossils in the nucleus. *Curr. Biol.* **6:** 128–129.

Pichersky, E., and Tanksley, S. D. (1988). Chloroplast DNA sequences integrated into an intron of a tomato nuclear gene. *Mol. Gen. Genet.* **215:** 65–68.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuschi, R., Horn, G. T., Mullis, K. B., and Ehrlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239:** 487–491.

Shay, J. W., and Werbin, H. (1992). New evidence for the insertion of mitochondrial DNA into the human genome: Significance for cancer and aging. *Mutat. Res.* **275:** 227–235.

Sunnucks, P., and Hales, D. F. (1996). Numerous transposed sequences of mitochondrial cytochrome oxidase I-II in aphids of the genus *sitobion* (Hemiptera: Aphidae). *Mol. Biol. Evol.* **13:** 510–524.

Tsuzuki, T., Nomiyama, H., Setoyama, C., Maeda, S., and Shimada, K. (1983). Presence of mitochondrial-DNA-like sequences in the human nuclear DNA. *Gene* **25:** 223–229.

Wallace, D. C., Stugard, C., Murdock, D., Schurr, T., and Brown, M. D. (1997). Ancient mtDNA sequences in the human nuclear genome: A potential source of errors in identifying pathogenic mutations. *Proc. Natl. Acad. Sci. USA* **94:** 14900–14905.

Walter, M. A., Spillett, D. J., Thomas, P., Weissenbach, J., and Goodfellow, P. N. (1994). A method for constructing radiation hybrid maps of whole genomes. *Nat. Genet.* **7:** 22–28.

Zischler, H., Gelsert, H., von Haeseler, A., and Pääbo, S. (1995). A nuclear fossil of the mitochondrial D-loop and the origin of modern humans. *Nature* **378:** 489–492.