Pharm Res (2011) 28:2863–2870
DOI 10.1007/s11095-011-0527-1

RESEARCH PAPER

Trial and Error: How the Unclonable Human Mitochondrial Genome Was Cloned in Yeast

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Received: 9 February 2011 / Accepted: 29 June 2011 / Published online: 9 July 2011

ABSTRACT

Purpose Development of a human mitochondrial gene delivery vector is a critical step in the ability to treat diseases arising from mutations in mitochondrial DNA. Although we have previously cloned the mouse mitochondrial genome in its entirety and developed it as a mitochondrial gene therapy vector, the human mitochondrial genome has been dubbed unclonable in *E. coli*, due to regions of instability in the D-loop and $tRNA^{Thr}$ gene.

Methods We tested multi- and single-copy vector systems for cloning human mitochondrial DNA in *E. coli* and *Saccharomyces cerevisiae*, including transformation-associated recombination.

Results Human mitochondrial DNA is unclonable in *E. coli* and cannot be retained in multi- or single-copy vectors under any conditions. It was, however, possible to clone and stably maintain the entire human mitochondrial genome in yeast as long as a single-copy centromeric plasmid was used. D-loop and $tRNA^{Thr}$ were both stable and unmutated.

Conclusions This is the first report of cloning the entire human mitochondrial genome and the first step in developing a gene delivery vehicle for human mitochondrial gene therapy.

KEY WORDS gene therapy · human mitochondrial DNA · mitochondrial disease · unclonable · yeast

ABBREVIATIONS

ARS	autonomous replicating sequence
BAC	bacterial artificial chromosome
D-loop	displacement loop
mtDNA	mitochondrial DNA
PAC	P1 phage artificial chromosome
TAR	transformation-associated recombination
TB	terrific broth

INTRODUCTION

Human mitochondria are small 0.5–1 μm cytoplasmic organelles, present in 10–100 copies per somatic cell, each containing several autonomously replicating mitochondrial DNA genomes (mtDNA) under close nuclear control. More than 50 pathogenic mutations and rearrangements have been identified in the 16.6 kb human mtDNA (1), which are together responsible for a disproportionately high incidence of mitochondrial disease of between 1/11,000–1/15,000 in NE England (2,3). This is surprisingly high, given that mtDNA only encodes 13 peptides, 2 rRNAs and 22 tRNAs, with the vast majority of mitochondrial proteins encoded in the nucleus and imported into mitochondria. Mitochondrial disease phenotype is dependent on mtDNA heteroplasmy, where disease manifests in cells carrying more than

Electronic Supplementary Material The online version of this article (doi:10.1007/s11095-011-0527-1) contains supplementary material, which is available to authorized users.

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a certain threshold percentage of mutated mtDNA copies, resulting in a mosaic pattern of expression (4). Diseases resulting from mtDNA mutations are thus significant pharmacological targets, particularly for gene therapeutic approaches (5,6). However, interventions to the mitochondrion are complicated by problems of delivery to the mitochondrion and the lack of human mitochondrial vector systems to date (5–7). One of the main reasons for this appears to be an inability to clone the entire human mtDNA (8), despite the fact that mouse mtDNA, of the same size and coding for the same RNAs and peptides, is relatively amenable to cloning.

We and others cloned and modified the entire mouse mitochondrial genome in *E. coli* over 15 years ago with a gene therapeutic approach in mind (8–11). In our approach, the nuclear encoded ornithine transcarbamylase gene was re-coded for mitochondrial expression and placed between two tRNAs in the mouse mitochondrial genome (11). At the time, we had some difficulty in cloning and modifying the mouse mtDNA in *E. coli* using standard techniques and had to resort to a shuttle cloning technique, using homologous recombination in *Saccharomyces cerevisiae* to establish a circular clone, before shuttling back to *E. coli* for conventional DNA preparation (9). This approach appears to stabilise mouse mtDNA sufficiently to obtain stable full-sized clones and also enabled us to construct several smaller minicircle mtDNA gene therapy constructs (10). More recently, we were able to demonstrate uptake of one of these 5.8 kb minicircle mouse mitochondrial gene therapy vectors into mitochondria (12). Given the burden of mitochondrial disease in humans, and the requirement for a human mtDNA vector to address a gene therapeutic approach in these diseases, we next turned our attention to cloning the entire human mtDNA.

The discrepancy in clonability of the entire human mitochondrial genome compared to its closely related neighbour mouse mtDNA was elegantly demonstrated in an experiment by Tapper *et al.* (8). A combination of 95% linearised human mtDNA with 5% linearised mouse mtDNA was ligated to linearised pBR322 and used to transform *E. coli* cells. The only positive clones obtained from this ligation mixture were full-length mouse mitochondrial DNA, with no detectable clones containing any human mitochondrial DNA (8). Mita and colleagues later reported the region of instability to be within the $tRNA^{Thr}$ region (13,14). We were also unable to obtain *E. coli* clones following direct linearization and ligation of full-length human mtDNA to a multicopy *E. coli* vector. Together, these data led us to the conclusion that although mouse mtDNA could be cloned in a multicopy *E. coli* plasmid such as pBR322, or the shuttle plasmid pRS316, this was not likely to be an effective strategy for cloning human mtDNA. In this paper, we have therefore explored different vectors, hosts, host strains and methodologies for cloning human mtDNA and report the generation of stable full-length human mtDNA clones in *S. cerevisiae*.

MATERIALS AND METHODS

Preparation of Human mtDNA

Human mtDNA was isolated from surplus blood or pooled platelet preparations using a method adapted from that of Shuster *et al.* (15). Whole blood was suspended in 3.8% sodium citrate to avoid coagulation. Red blood cells were extracted by 2x centrifugation at 300 g for 20 min followed by centrifugation at 700 g to precipitate platelets. These were resuspended in 10 ml platelet lysis buffer (120 mM NaCl, 10 mM Tris (pH 8.0), 50 mM EDTA), adding 2.5 ml 10% SDS and 120 µl 20 mg/ml proteinase K, and left overnight at 55°C. DNA was phenol/chloroform prepared, precipitated and resuspended in TE buffer as previously described (9).

Cloning in *E. Coli*

Max. efficiency STBL2^{TM} *E. coli* cells (Gibco BRL) were transformed by electroporation (Biorad Gene pulser) with ligation mixes of 1–5 µl of 5:1 molar ratios of human mtDNA and pRS316 according to manufacturer's instructions. All bacterial preparation was performed at 30°C in Terrific Broth (TB) to stabilise large constructs as previously described (9). For shuttle cloning from yeast to *E. coli*, 1.5 µl of DNA was used as previously applied to the mouse mtDNA cloning (9) using the strains STBL2, DH10B, DH5α (Gibco BRL) or SURE (Stratagene).

Construction of Vectors for Homologous Recombination in Yeast

Two regions of homology to 500 bp regions of the human mtDNA between bases (8461–8997) (HR85) and (8998–9524) (HR95), separated by the unique BsiWI restriction site, were chosen for amplification and cloning.

The HR85 fragment was PCR amplified using primers 85F 5′CAAGATCTACCACCTACCTCCCTCAC CAAAGCC3′ and 85R 5′CACTCGAGTCGACG TACGGCCAGGGCTATTGG3′, which added a BglII site to the 5′ end and a SalI and XhoI site to the 3′ end. The HR95 fragment was PCR amplified using primers 95F 5′CAGAATTCCGTACGCCTAACCGCTAAC 3′, 95R 5′CAAGATCTAGGCTGGAGTGGTAAAAGGC 3′, which added an EcoRI site at the 5′ end and a BglII site at the 3′ end. Both fragments were individually TA cloned into the cloning vector pCR2.1 to create pCR 85 and pCR

95. Digestion of pCR 85 with SalI and BglII frees the HR85 fragment, whilst digestion of pCR 95 with EcoRI and BglII frees the HR95 fragment.

Both fragments were then simultaneously inserted into the SalI/EcoRI sites of the yeast/bacterial shuttle vector pRS316 to create pRSHR89. This results in a standard homologous recombination vector, which contains an ARS, an *ura3* gene as well as a yeast centromere, resulting in stable low copy number propagation in yeast on media lacking uracil. Note that the HR85 and HR95 fragments are now in reverse order in pRS316 (HR95, HR85) but the same orientation. Thus, when pRS89 is digested with BglII, it linearises to become a homologous acceptor for the human mtDNA (Fig. 1). The HR 95/HR85 regions and insertion sites into pRSHR89 were fully sequenced using primers 85F, 85R, 95F, 95R and BglIIcover 5′GCCTTCGATACGGGATAATC3′, and all homologous regions corresponded to the Cambridge reference sequence of human mtDNA (16). The TAR cloning vector for homologous recombination of human mtDNA was derived from pRSHR89 by the removal of the HR95/HR85 region at the EcoRI/XhoI sites in pRSHR89 and re-insertion into the yeast replication deficient vector pVCHP1 at the corresponding sites. Primers PVC85Cover 5′CTTCTTACCACAAGGCACAC 3′, and PVC95Cover 5′GTGTAGAGGGAAGGTTAATG 3′ were used to sequence the insertion sites of the 85/95 human mtDNA region of homology in pTARHR89.

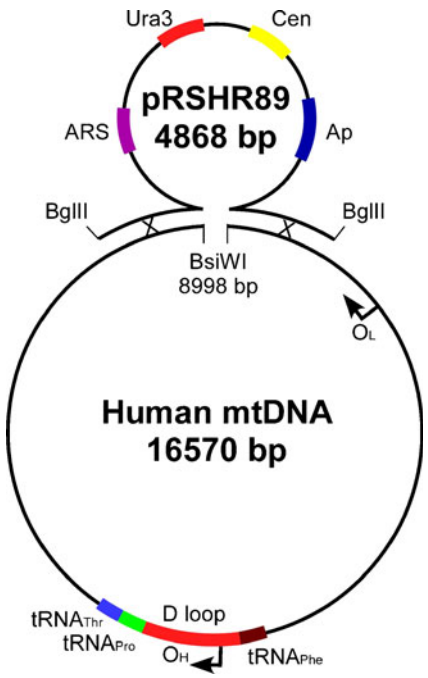

Fig. 1 The successful homologous recombination cloning strategy. The construct pRSHR89 possesses two arms of homology to human mtDNA. Recombination of this BglII linearised construct with BsiWI linearised human mtDNA in yeast permits isolation of yeast clones containing both pRS316 and the entire human mtDNA. A very similar strategy was attempted with homologous recombination of either pTARHR89 or YEpHR89 and human mtDNA. Also highlighted is the triple-stranded D-loop region and flanking tRNAs, particularly the $tRNA^{Thr}$ that has previously been reported to be the source of cloning instability in human mtDNA (13,14).

The pTARHR89 plasmid is non-permissive in yeast and should not allow growth of yeast transformed with unrecombined plasmid on media lacking histidine. The addition of the ARS- like sequence from human mtDNA to pTARHR89 by recombination should permit growth of yeast transformed with these clones on media lacking histidine.

The multicopy yeast plasmid for homologous recombination of human mtDNA was derived from YEp24, by removal of the HR95/HR85 fragment from pRSHR89 by digestion with XmaI and SalI, and transferred to the corresponding sites in YEp24 to create the plasmid YEpHR89. Both pTARHR89 and YEpHR89 were checked for insert integrity by multiple restriction digestions.

Homologous Recombination in *S. Cerevisiae*

Homologous recombination between BsiWI linearised human mtDNA and BglII linearised pRSHR89 in a slightly greater than 1:1 molar ratio was carried out by incubating 100–200 ng of ligation mixture and 5 µg of carrier plasmid DNA (plasmid pGEM5Zf) with VL6 48 yeast spheroplasts made as previously described (9). Recombinants were selected on basic drop-out plates (0.68% yeast nitrogen base [without amino acids], 2% D-glucose, 0.077% Casamino acids without uracil [CSM-URA], 2% bactoagar [pH adjusted to 5.8 with 10 N NaOH]) or richer $aAHC^{Ura-Trp+}$ dropout plates as previously described (9). The procedure differed for pTARHR89 recombination by the use of basic drop-out plates as above lacking histidine (CSM-HIS) and for YEpHR89 by the use of strain YPH925 or VL6 48 (both generous gifts from Dr. Amanda McGuigan), and selection on $aAHC^{Ura-Trp+}$ drop-out plates.

Screening for Human mtDNA Clones

Colony hybridization and Southern hybridization were carried out as previously described (9). DNA probes of a 1.3 region including the human mtDNA D-loop region were made by PCR amplification with primers Dseq5 5′ CTTCACAACAATCCTAATCC 3′ and HDL2 5′ CCCGTCTAAACATTTTCAGTG 3′ and radiolabelled with [α-^{32}P]dCTP (Amersham), using the Megaprime Labelling kit (Amersham).

Sequence Data and Alignment

Automated sequencing was carried out using the Big Dye cycle sequencing technology (Applied Biosystems) and run on an Applied Biosystems 3730 DNA analyzer. Chromatograms were checked for fidelity on BioEdit, and contigs of sequenced regions were constructed using Seqman (DNASTAR Inc.) and aligned with the human mtDNA reference sequence using Megalign (DNASTAR Inc.) to obtain the final sequence of pRShmt (clone 1B9). This was checked against known human mtDNA polymorphisms reported on the MITOMAP database (www.mitomap.org). The full 21438 bp sequence of pRShmt clone 1B9 is available from Genbank accession number JN160804. PCR and sequencing primers used to sequence pRShmt are provided in Supplementary Table 1.

Yeast DNA Preparation

The method for DNA preparation for shuttle cloning was performed as previously described (9). Large-scale 4 L yeast cultures for preparation of DNA from pRShmt clones were scaled up from this method 160-fold and were adapted from the *E. coli* DNA Gigaprep kit (Qiagen) in exactly the same way.

RESULTS

Human mtDNA Not Clonable in *E. Coli* in Multi-Copy Vectors or Single-Copy PAC Vectors

Having failed to obtain *E. coli* clones following direct linearization and ligation of full-length human mtDNA in a multi-copy *E. coli* vector, we investigated several *E. coli* P1-Phage Artificial Chromosomes (PAC) clones that were generous gifts from Dr. Pannos Ioannou and had been obtained by ligation of linearised pCYPAC2 vector to BamHI linearised human mtDNA (17).

PACs are maintained as autonomously replicating single-copy clones and have improved stability over multi-copy vectors. Unfortunately, all clones showed BglII digestion patterns consistent with recircularisation of the 16.8 kb BamHI pCYPAC2 vector fragment to itself. Southern hybridisation to a D loop probe showed no detectable human mtDNA (Data not shown).

Human mtDNA Does Not Contain Yeast Autonomous Replication Sequence and Cannot Be TAR Cloned

One reason for instability in human mtDNA could be that regions within the genome function as origins of replication in *E. coli*, or that they function as Autonomous Replication Sequences (ARS) in yeast. Given the instability we observed in *E. coli*, we constructed a Transformation Associated Recombination (TAR) cloning vector (18,19) for cloning in the yeast *S. cerevisiae*. This vector lacks an ARS region, required for yeast replication, which is provided by the insert, thus permitting vector propagation. Human mtDNA D loop contains a close match to the 11 bp consensus ARS sequence (Table I) (20,21), and close matches are known to function successfully (22). The D loop region may also provide the required DNA unwinding regions.

We constructed the *His3* TAR cloning vector pTARHR89 by modifying pVCHP1 (23) to include arms of homology to 500 bp regions flanking the unique BsiWI site in human mtDNA. Despite many attempts, we never obtained any clones, whilst control recombinations were always successful (not shown). This suggests that human mtDNA does not contain an origin of replication in yeast (a functional ARS sequence); therefore, although it cannot be cloned by TAR cloning, it is likely to be able to be propagated in yeast successfully.

Human mtDNA Successfully Cloned in Yeast as pRShmt by Homologous Recombination

We subsequently constructed a homologous cloning vector pRSHR89 based on the *Ura3* yeast centromeric vector pRS316 that we had previously used to clone the entire mouse mitochondrial genome (9–11) using the same arms of homology to human mtDNA. This vector is maintained in yeast at 1–2 copies/cell, faithfully segregating with chromosomal replication (24).

We recombined pRSHR89 with human mtDNA in yeast spheroplasts as described (Fig. 1). From 420 colonies analysed, 12 hybridised to the human mtDNA D-loop region by Southern blot (not shown). Due to the single-copy nature of centromeric vectors in yeast, and the presence of several contaminating circular DNAs in yeast including circular chromosomes, the 2 μm circle and yeast mtDNA, it is not possible to clearly visualise a purified plasmid construct on an agarose gel from a yeast DNA preparation. We subsequently digested the 12 positive clones of pRShmt (21.4 kb) alongside human mtDNA (16.6 kb) with XbaI

Table I A Putative ARS Consensus Region in the Human mtDNA D-loop Region

ARS Consensus	A/T T T T A T Pu T T T A/T
Human mt D-loop	T T T T A T C T T T T

The consensus ARS sequence functions as an initiation site for replication in yeast. The human mtDNA contains a very similar sequence in the D-loop regulatory region (398–408 bp), differing only by a cytosine in place of the central purine residue. Sequences that are not entirely consensus may also function successfully as ARS sites.

(Fig. 2A) and EcoRI (Fig. 2B) and performed a Southern hybridisation to total human mtDNA. Eight clones showed bands at sizes corresponding to a full-length human mtDNA insert in pRS316; clones 5, 6, 8, 9 had additional bands. To confirm that we had indeed cloned the unstable regions of human mtDNA without gaining mutations, we PCR amplified and sequenced the entirety of clone 1 (pRShmt clone 1B9; Genbank accession JN160804). The $tRNA^{Thr}$ region was reported by Mita and colleagues to be the source of instability in their human mitochondrial clones (13,14) and was later characterised with unclonable *E. coli* sequence mutation hotspots at 15900–15904 and 15923–8 within the cloverleaf structure of the tRNA (25). We found no sequence variations in the $tRNA^{Thr}$ gene cloned in pRShmt to either the human mtDNA Cambridge reference sequence (16) or purified BWB human mtDNA (Fig. 3).

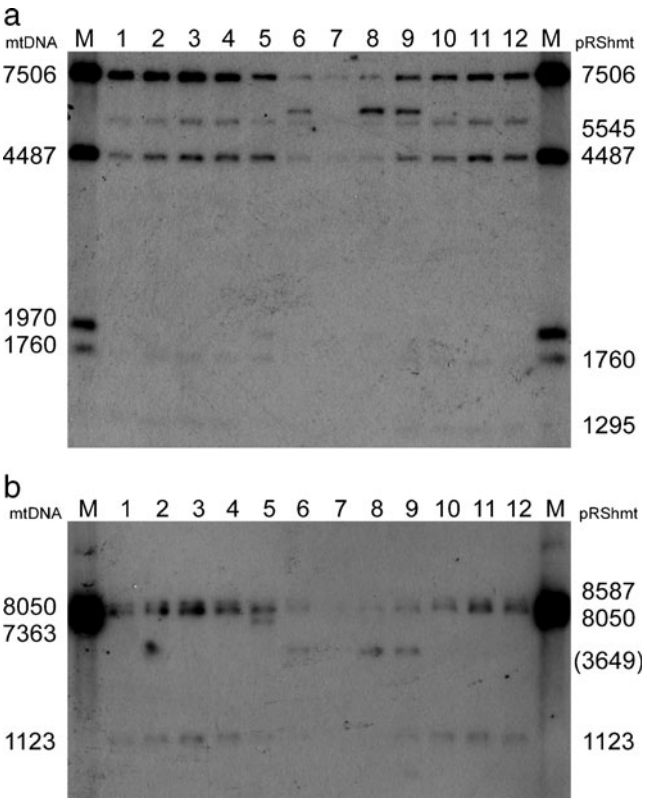

Fig. 2 Restriction digests and Southern hybridisation of pRShmt clones to human mtDNA. (**a**) Yeast clones 1–12 of pRShmt and human mtDNA (M) were digested with XbaI. These were then hybridised to total human mtDNA. The numbers on the left give the expected band sizes for digestion of human mtDNA with XbaI, whilst those on the right correspond to those expected for pRShmt. The bands obtained are all of the correct sizes, with the exception of the extra band >5545 in clones 6, 8, 9 and one at 1970 in clone 5. The construct pRShmt thus appears to contain the full sized human mtDNA. (**b**) Yeast clones 1–12 of pRShmt and human mtDNA (M) were digested with EcoRI. These were then hybridised to total human mtDNA. The numbers on the left give the expected band sizes for digestion of human mtDNA with EcoRI, whilst the bands expected for pRShmt are listed on the right. All bands obtained for both pRShmt and human mtDNA appear to be correct. Band (3649) in pRShmt is not expected to hybridise, as it contains only pRS316 sequences. An extra band appears in clones 6, 8, 9 at a height >3649. A band also appears in clone 5 at <8050. All other clones appear to contain complete pRShmt.

Table II presents a list of the 13 single bp polymorphisms found in the pRShmt (1B9) sequence compared to the Cambridge reference sequence. All polymorphisms can be found in Mitomap with the exception of the C6237A mutation which has not been previously described and leads to a L-M coding change in the ND2 gene. None pertain to putative sequence instability reported by other groups (13,14,25) nor to disease mutations.

pRShmt Cannot Be Stably Shuttle Cloned from *S. Cerevisiae* into *E. Coli*

Despite many attempts to shuttle clone the 21.4 kb pRShmt construct prepared from yeast back into several different strains of *E. coli*, we were never able to shuttle clone this plasmid successfully, despite the fact that control yeast preps of the 22 kb pRSmtOTC (the entire mouse mitochondrial genome+ornithine transcarbamylase gene) were viable as shuttle clones in *E. coli*. More than 200 colonies were analysed but none containing full-length human mtDNA.

Human mtDNA Cannot be Cloned into Multicopy Yeast Plasmids

We also investigated the stability of pRShmt in multi-copy vectors in *S. cerevisiae*, which would increase plasmid yield.

The well-characterised 10–40 copy 2 μm derived plasmid YEp24 (26) was unable to support clones of human mtDNA in yeast. We constructed the YEpHR89 homologous recombination vector by cloning the arms of homology to the human mtDNA from pTARHR89 into Yep24 and recombining this vector with human mtDNA as described. Following analysis of more than 300 colonies, we have never obtained positive clones hybridising to human mtDNA.

The 50–100 copy 2 μm pJDB219 (27), which has a defective *leu2* gene (*leu2-d*), provided an abnormal restriction pattern and was unable to complement the YPH925 yeast strain on leucine drop-out media, suggesting that it had undergone rearrangement due to the presence of repeat sequences within the 2 μm circle. We did not pursue this vector further.

DISCUSSION

Despite numerous attempts to clone the entire human mtDNA in several different *E. coli* cloning hosts, we and

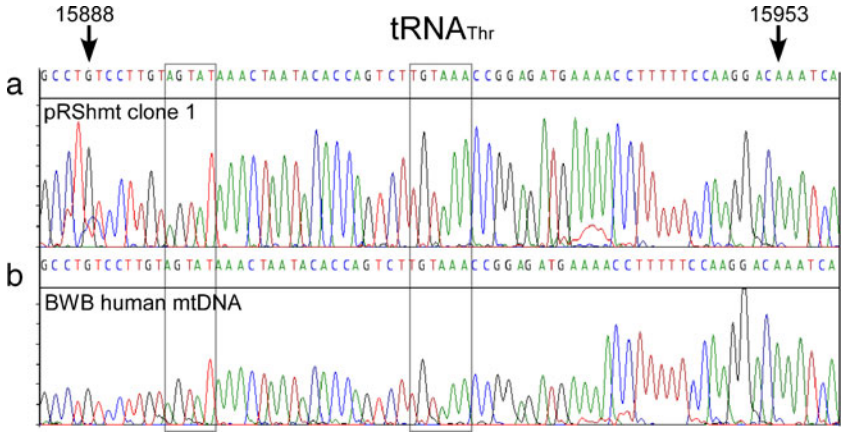

Fig. 3 Comparative sequence of the tRNA^{Thr} gene from pRShmt and BWB human mtDNA. The tRNA^{Thr} gene is believed to contain sequences responsible for the instability in *E. coli* of human mtDNA clones (13,14). The tRNA^{Thr} gene was PCR amplified from both (**a**) pRShmt yeast clone 1 and (**b**) BWB human mtDNA and sequenced. There were no sequence differences observed between pRShmt, BWB human mtDNA or the published sequence of the human mtDNA tRNA^{Thr} gene (16). Thus, pRShmt contains an intact, unmutated tRNA^{Thr} gene. The entire pRShmt clone 1 (1B9) was sequenced (Genbank accession JN160804), including the D-loop and flanking tRNAs with polymorphisms described (Table II).

others never achieved success using this method. This is apparently not uncommon (8,13,14) and confirms our previous problems encountered with cloning the entire mouse mtDNA (9–11). Attempts to clone human mtDNA in a single copy *E. coli* PAC vector, thus improving stability (17), were also unsuccessful. Although we were able to clone human mtDNA in *S. cerevisiae* using a method we developed for cloning mouse mtDNA (9), unlike mouse mtDNA, we were unable to shuttle clone human mtDNA back into an *E.coli* host, despite attempts in several different host strains.

The sequence region responsible for the instability in human mtDNA in *E. coli* might be expected to be the D-loop region because of its high potential for secondary structure; however, the "unclonable" region of human mtDNA is reported to be in the tRNA^{Thr} region, adjacent to the D-loop (13,14). One possible mechanism for instability in *E. coli* could be the creation of an alternative plasmid origin of replication by this region. The rat mitochondrial origin of replication, for example, has been shown to act as a plasmid origin of replication in bacteria, under conditions when the

Table II Comparative Sequence Polymorphisms in Human mtDNA

Human mtDNA Cambridge reference sequence	Nucleotide change in pRShmt (clone 1B9)	Codon change	Region affected	Polymorphism listed in MITOMAP
152	T-C	None	D loop	√
263	A-G	None	D loop	√
311–315	5xC-6xC	None	D loop	√
750	A-G	None	12S rRNA	√
1438	A-G	None	12S rRNA	√
3010	G-A	None	16S rRNA	√
4769	A-G	M-M	ND2	√
6040	A-G	N-S	COX1	√
6237	C-A	L-M	COX1	x
6410	C-T	I-I	COX1	√
8860	A-G	T-A	ATP6	√
15326	A-G	T-A	CYTB	√
16519	T-C	None	D loop	√

The entire 21438 bp pRShmt (1B9 clone) was PCR amplified and sequenced (genbank accession JN160804), including the 16569 bp human mtDNA, tRNAPro and tRNAThr. The tRNA regions were all identical to the published sequence. Polymorphisms and codon changes were all listed in MITOMAP, with the exception of the C6237A change, and none were associated with disease or instability mutations (16). Four polymorphisms were evident in the D-loop region itself in both pRShmt and in BWB human mtDNA. A263 is a rare polymorphism (16) and shows that the regions most likely to be unstable in pRShmt are present without mutation.

bacterial origin is non-permissive (28), although no data is presented on the stability of these bacterial clones under permissive conditions.

Transformation associated recombination (TAR) cloning (18,19) provided a method for reducing the background of unrecombined pTARHR89 colonies by the simple stratagem of making the cloning plasmid (without an insert) non-viable in yeast through the lack of an autonomously replicating sequence (ARS), which would be provided by the insertion of human mtDNA. It also provided an opportunity to see if the human mtDNA had yeast origin of replication competent regions. This approach was supported by the successful transformation of yeast spheroplasts with mitochondrial DNA of *Xenopus laevis*, which was then maintained in these cells as a high copy number unstable extrachromosomal element, suggesting the presence of an ARS or ARS-like sequence in *Xenopus* mtDNA (29). The failure to clone human mtDNA by this method suggests that the sequence in the human mitochondrial D-loop, which differs from the yeast ARS consensus sequence (30) by only a single mismatch, was not functional. Whilst sequences that differ slightly from the consensus may still function as ARS sequences (22), regions flanking the ARS may also be important for replication by providing DNA unwinding regions and further protein binding sites (31).

It is also possible that the presence of secondary structure in the D-loop region made the ARS unavailable for protein binding and thus initiation of replication in yeast cells.

Clearly this does not rule out the possibility that human mtDNA may contain a bacterial origin of replication.

The cloning of the entire human mtDNA into pRSHR89 was remarkable by its simplicity and also the relatively low levels of screening required to obtain full-length human mtDNA clones (pRShmt). Although there are problems with isolation of pure plasmid DNA from yeast, the restriction digests and sequence information provide clear evidence of the presence of full-length, unrearranged human mtDNA clones with no deletions or non-polymorphic mutations in the D-loop or $tRNA^{Thr}$ regions. Mita *et al.* previously reported mutations in the highly unstable $tRNA^{Thr}$ region when cloned in *E. coli* (13,14), which are not present in these yeast clones.

Despite the stability of pRShmt in *S. cerevisiae*, it was not possible to obtain clones of this plasmid in *E. coli* by shuttle transformation. The instability of human mtDNA clearly far exceeds that of the mouse mtDNA, and a genuinely toxic sequence, whether blocking replication or causing plasmid recombination, might be expected to remain incompatible with propagation in *E. coli*, even after stabilisation and amplification of the DNA by cloning in yeast. Although large volumes of yeast culture may be prepared for subsequent pRShmt purification, the yields from centromeric plasmids are very low, and purification is complicated by the presence of chromosomal DNA and a number of low molecular weight yeast extrachromosomal elements.

The alternative approach of improving plasmid DNA yield by recloning the mtDNA into a higher copy number yeast vector was also unsuccessful.

We were unable to use pJDB219 due to rearrangements in the construct, and although we were able to create a construct for homologous recombination using the multi-copy yeast 2 μm plasmid YEp24 (26), we have not yet revealed any full-sized human mtDNA clones. It is possible that human mtDNA is not clonable in yeast using a multi-copy 2 μm derived vector such as YEp24. This would be in keeping with previous reports of the non-clonability of human mtDNA in yeast using this vector (Wallace, personal communication).

This suggests two separate mechanisms of instability for human mtDNA. First, there is very clearly a sequence-specific effect in *E. coli* that is independent of size. Second, a combination of vector size and specific regions of sequence instability probably also affects cloning in yeast. As bacterial vectors are developed further, and the sequences responsible for the instability of this genome are further characterised, it may be possible to select an appropriate genetic background for stable cloning and maintenance of the human mtDNA in *E. coli*. A systematic search for the precise sequences responsible for instability would also be useful. The single-copy yeast clone of human mtDNA provides us with an interim construct with which to further investigate mitochondrial gene therapeutic strategies and the source of bacterial instability.

CONCLUSION

In summary, we have cloned the unclonable entire human mtDNA in a single-copy centromeric plasmid in *S. cerevisiae* and shown that the entire human mtDNA, including the highly unstable $tRNA^{Thr}$ region (13,14) and adjacent D-loop region, are intact and un-mutated. Although these clones are stable in themselves, human mtDNA plasmids demonstrate instability on shuttle cloning to any *E. coli* host, even as circular plasmids.

The reason for the instability remains unclear but may have different causes in different host organisms. The plasmid is no larger than that of the entire mouse mtDNA that we successfully shuttle cloned into *E.coli* (9,10), but is not maintained in a single-copy PAC vector in *E. coli*. The $tRNA^{Thr}$ region may function as a second origin of replication in *E. coli*, or it could be that this region or the triplex DNA structures formed in mtDNA and some peculiarity of human mtDNA make it unclonable in *E.coli*. A secondary problem is encountered with an inability to

clone human mtDNA in multi-copy yeast plasmids, suggesting a separate size/stability issue in yeast.

Maximisation of plasmid stability by the use of single-copy plasmids and the more permissive yeast host organism has permitted the cloning of the previously unclonable human mitochondrial genome. The cloning of the pRShmt construct is the first step in development of novel gene therapy approaches to treating diseases caused by mutations in human mitochondrial DNA.

ACKNOWLEDGMENTS & DISCLOSURES

This work was supported by the Cystic Fibrosis Trust Muller Bequest to CC. BWB, AYL and AS are supported by the UK society for Mucopolysaccharide diseases and the Manchester Biomedical Research Centre.

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