Revisiting the mouse mitochondrial DNA sequence

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ABSTRACT

The existence of reliable mtDNA reference sequences for each species is of great relevance in a variety of fields, from phylogenetic and population genetics studies to pathogenetic determination of mtDNA variants in humans or in animal models of mtDNA-linked diseases. We present compelling evidence for the existence of sequencing errors on the current mouse mtDNA reference sequence. This includes the deletion of a full codon in two genes, the substitution of one amino acid on five occasions and also the involvement of tRNA and rRNA genes. The conclusions are supported by: (i) the re-sequencing of the original cell line used by Bibb and Clayton, the LA9 cell line, (ii) the sequencing of a second L-derivative clone (L929), and (iii) the comparison with 12 other mtDNA sequences from live mice, 10 of them maternally related with the mouse from which the L cells were generated. Two of the latest sequences are reported for the first time in this study (Balb/cJ and C57BL/6J). In addition, we found that both the LA9 and L929 mtDNAs also contain private clone polymorphic variants that, at least in the case of L929, promote functional impairment of the oxidative phosphorylation system. Consequently, the mtDNA of the strain used for the mouse genome project (C57BL/6J) is proposed as the new standard for the mouse mtDNA sequence.

INTRODUCTION

The mtDNA from the cell line LA9, a derivative clone of the L cell strain, was the first mouse mtDNA to be sequenced (1) and it has been considered as the standard reference (named hereafter as LA9[1981]). The parent L strain was generated in the 1940s from normal subcutaneous aerolar and adipose tissue of a 100-day-old male C3H/An mouse (2). However, since its publication, a number of mouse mtDNA full sequences showing important discrepancies with the LA9 reference have been reported (Table 1). These discrepancies could be due to existing polymorphisms in the mouse populations, acquired mutations during the lifetime in culture of the LA9 cell line or sequencing errors. Since mouse models for human mtDNA-linked diseases are starting to be generated (3–5), and because this sequence is used for phylogenetic studies including comparative analysis to estimate the potential pathogenicity of human mtDNA variants, the clarification of these uncertainties is of major interest.

Information obtained from live animals is available for the full mtDNA sequence of 10 different *Mus musculus* strains. These are: (i) C3H/He and six other strains belonging together to the group B (Castle's mice) of mouse-inbred strains (6) [A/J (7), NZB/B1NJ (8), AKR/J and its derivative strains SAMR1, SAMP1 and SAMP8]; (ii) a congenic derivative from the group A (Swiss mice) NOD/LtJ inbred strain, NOD.NON-H2[nb1]/LtJ (7); (iii) the spontaneous mutant strain SKH2/J (7); and (iv) the Mil P mouse strain, descendant from a wild female mouse caught near Pavia, Italy (8). We report here another two mtDNA sequences of mouse-inbred strains, Balb/cJ, a group B inbred strain (6), sequenced at Zaragoza University, and C57BL/6J, a group E inbred strain (6), obtained at the Sanger Institute from the same source of DNA used for the sequencing of the mouse nuclear genome. In addition, we have performed the full sequencing of the LA9 cell line mtDNA (called hereafter LA9[2002]) and an additional L-derived cell clone (L929) mtDNA.

MATERIALS AND METHODS

Numbering of the oligonucleotides used

For standardization we have referred the numbering of the starting and ending nucleotides of the different primers used in this research to the C57BL/6J mtDNA.

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[+]AJ489607, AY172335 and AJ512208

Table 1. Conflicting positions in the LA9 mouse mtDNA reference sequence

Position[a]	Gene	Cell lines		Related mouse strains											Unrelated mouse strains		Proposed reference[b]
		LA9[1981] 16 295	L929 16 301	LA9[2002] 16 300	C3H/He 16 301	AKR/J 16 300	Balb/cJ 16 300	A/J 16 301	NOD/LtJ 16 300	SKH2/J 16 300	SAMR1 16 299	SAMP1 16 299	SAMP8 16 299	NZB/B1NJ[a] 16 303	Mil P[a] 16 303	C57BL/6J 16 299
1767e	rRNA16S	a	g	g	g	g	g	g	g	g	g	g	g	g	g	g
2256	rRNA16S	t	t	t	t	t	t	t	t	t	t	c	c	t	t	t
3815e	tRNA[Gln]	t	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3824e	tRNA[Gln]	—	t	t	t	t	t	t	t	t	t	t	t	t	t	t
4012e	Nd2	a	g	g	g	g	g	g	g	g	g	g	g	g	g	g
4794	Nd2	t[c]	c	t[c]	c	c	c	c	c	c	c	c	c	c	c	c
5734e	CoI	c	t	t	t	t	t	t	t	t	t	t	t	t	t	t
5737e	CoI	t	c	c	c	c	c	c	c	c	c	c	c	c	c	c
6063	CoI	c	c/a[d]	c	c	c	c	c	c	c	c	c	c	c	c	c
6238e	CoI	g	a	a	a	a	a	a	a	a	a	a	a	a	a	a
6589	CoI	t	c[d]	t	t	t	t	t	t	t	t	t	t	t	t	t
9348	CoII	a	a	a	a	g	a	a	a	g	g	g	g	g	g	g
9461	Nd3	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c
9554e	Nd3	—	aaa	aaa	aaa	aaa	aaa	aaa	aaa	aaa	aaa	aaa	aaa	aaa	aaa	aaa
9818e	tRNA[Arg]	—	aa	-a	ta	-a	-a	aa	-a	-a	—	—	—	aa	aa	—
9856e	tRNA[Arg]	c	a	a	a	a	a	a	a	a	a	a	a	a	a	a
10 059e	Nd4L	—	atc	atc	atc	atc	atc	atc	atc	atc	atc	atc	atc	atc	atc	atc
10 841	Nd4	g	g	g	g	g	g	g	g	g	g	g	a	g	g	g
11 175	Nd4	a	a	a	a	a	a	a	a	a	a	g	g	a	a	a
12 042	Nd5	c[c]	t	c[c]	t	t	t	t	t	t	t	t	t	t	t	t
13 046	Nd5	t	c[c]	t	t	t	t	t	t	t	c	c	c	t	t	t
13 879	Nd6	6 Cs	6/7 Cs[d]	6 Cs	6 Cs	6 Cs	6 Cs	6 Cs	6 Cs	6 Cs	6 Cs	6 Cs	6 Cs	6 Cs	6 Cs	6 Cs
15 823e	D-loop	t	—	—	—	—	—	—	—	—	—	—	—	—	—	—
16 119e	D-loop	a	c	c	c	c	c	c	c	c	c	c	c	c	c	c
16 240e	D-loop	a	—	—	—	—	—	—	—	—	—	—	—	a	a	—
Accession no.		J01420	AJ489607	AY339599	AB049357	AB042432	AJ512208	NA	NA	NA	AB042523	AB042524	AB042809	L07095	L07096	AY172335

The number under the name of each sequence indicates the mtDNA total length. NA, not available.
[a]The table shows differences in the mtDNA sequence at the indicated loci numbered according to Bibb et al. (1), the remaining positions are identical between all the sequences with the exception of the two more divergent ones (NZB and Mil P), which showed a high number of differences not indicated. The positions at which the LA9[1981] sequence is likely to be wrong are followed by an e.
[b]The heteroplasmic locus at position 5172 is not indicated.
[c]In culture, mutations acquired by LA9.
[d]In culture, mutations acquired by L929.
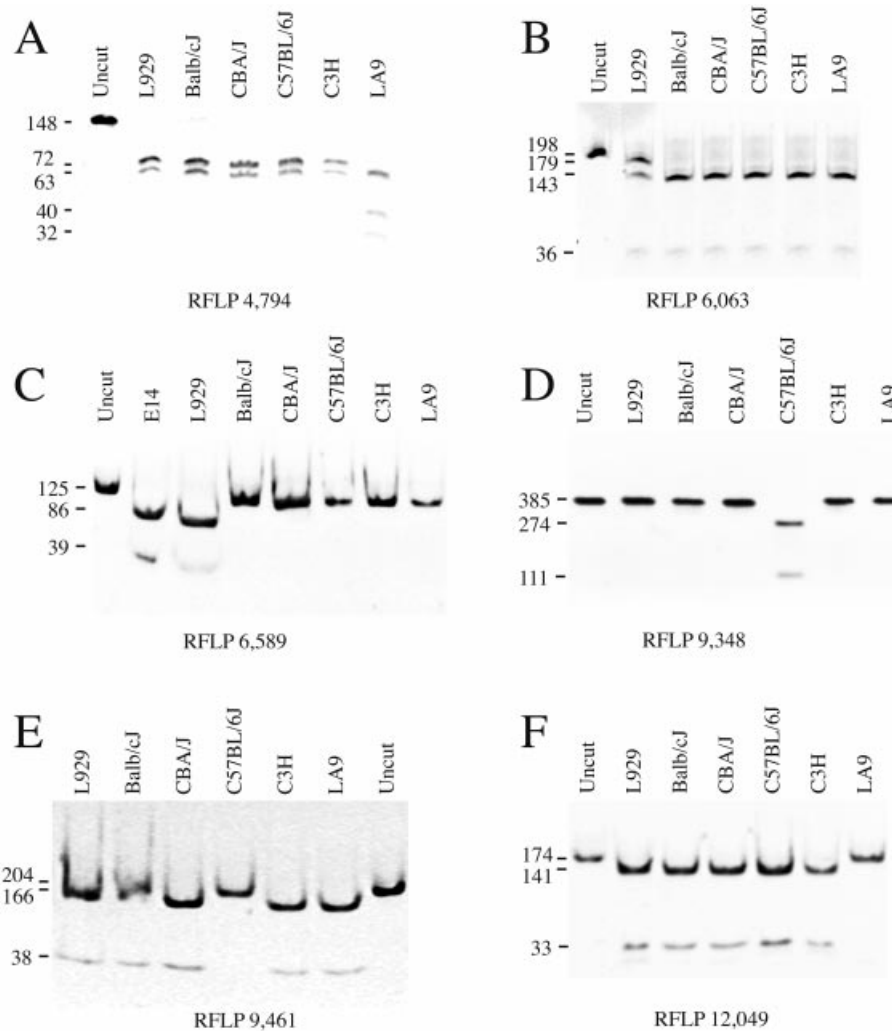[e]Highly polymorphic locus.

Figure 1. RFLP confirmation and degree of heteroplasmy of private mutations observed in LA9 (**A** and **F**) and L929 mtDNAs (**B** and **C**) and specific polymorphysms detected in C57BL/6J mtDNA (**D** and **E**). The number under each panel indicates the nucleotide position responsible for the RFLP. The amplification products and the different restriction enzymes used are described in Material and Methods.

Sequence analysis

The comparison of mtDNA sequences from the different mouse strains and cell lines was performed using BLAST (9).

mtDNA sequencing

Total DNA was isolated from cells by digestion with proteinase K in buffer TE (10 mM Tris, 1 mM EDTA, pH 7.5), containing 0.5% SDS and ribonuclease A, purified by extraction with phenol–chloroform–isoamyl alcohol and precipitated with ethanol. Seventeen overlapping segments of 1000–1500 bp covering the entire mtDNA were amplified by PCR, with the appropriate oligodeoxynucleotides. The PCR products were purified using the Rapid PCR purification system of Gibco BRL, and the purified double-stranded fragments were directly sequenced by the chain termination method, using nested primers.

Polymorphysm analysis

Quantification of the *CoI* 6589 mutation was achieved by RFLP analysis, and quantification of the *Nd6C* insertion was carried out by allele-specific termination of primer extension as described elsewhere (10).

To confirm the *CoI* A6063C mutation, RFLP analysis was carried out. Thus, a 198 bp fragment was amplified by PCR with the following primers: (i) Fw, GCATCTGTTCTGA-TTCTTTGGGCACCCAGAAGTTTAaATT (positions 6023–6062, forward primer carrying a mismatch indicated by the lower case a); (ii) Rev, TCTAATCCTACTGTGAATtTGT-GGTGGGCTC (positions 6190–6220, reverse primer carrying a mismatch indicated by the lower case t in order to create an internal restriction site).

The primer-generated mutations together with the C6063 wild-type version create two recognition sites for AcsI and produce three bands of 143, 36 and 19 bp upon digestion with this enzyme. The restriction site that produces the 143 and 36 bp bands is disrupted when the mutation is present and a new band of 179 bp appears. Therefore, an internal control for full digestion with AcsI is present in the analysis. Fragments were analyzed by electophoresis in a 5% polyacrylamide gel. Notice that the 19 bp band is lost in Figure 1B.

For the *CoIII* A9348G polymorphism analysis, a 385 bp fragment containing the 9348 site was amplified by PCR with the following primers: (i) Fw, CGAAACCACATAAATC-AAGCCC (positions 9072–9093); (ii) Rev, CTCTCTTCT-GGGTTTATTCAGA (positions 9435–9456).

The G9348 version generates a recognition site for AspI while this restriction site is disrupted when the polymorphism A9348 is present. Fragments were visualized by electrophoresis in a 2% agarose gel containing 0.5 μg/ml ethidium bromide.

The *Nd3* C9461T polymorphism was confirmed by RFLP analysis. Thus, a 204 bp fragment containing the 9461 site was amplified by PCR with the following primers: (i) Fw, TTCCAATTAGTAGATTCTGAATAAACCCAGAAGAGA-GTgAT (positions 9420–9460, forward primer carrying a mismatch indicated by the lower case g); (ii) Rev, AAATTT-TATTGAGAATGGTAGACG (positions 9600–9623).

The combination of the primer-generated mutation together with the C9461 wild-type version produces a recognition site for BclI. Thus, the presence of the T9461 polymorphism disrupts the restriction site. Fragments were analyzed by electrophoresis in a 5% polyacrylamide gel.

To check the *Nd2* 4794T polymorphism present in LA9[2002] a 148 bp fragment was amplified by PCR with the following primers: Fw, ACTCATAGCAATAATAGCTCTACTAAA-CCTATTCTTTaAT (positions 4753–4792, forward primer carrying a mismatch indicated by the lower case a); Rev, TAGGGTGGAAAATATTAGGTTGGG (positions 4877–4900).

This amplicon of 148 bp contains two restriction sites for SspI that produce three DNA fragments of 72, 63 and 13 bp. In addition, the combination of the PCR-generated mutation together with the T4794 version, present in the LA9[2002] cell line, generates a third recognition site for SspI while this restriction site is absent when the C4794 polymorphism is present. The new restriction site allows the 72 bp band to be cut giving rise to a 40 and 32 bp band. Notice that the 13 bp band is lost in Figure 1A. Fragments were visualized by electrophoresis in a 10% polyacrylamide gel.

To analyze the *Nd5* T12049C polymorphism present in LA9[2002] (equivalent to the 12048 in C57BL/6J) a 174 bp fragment containing this site was amplified with the following primers: Fw, CTGTAGCCCTTTTTGTCACATGATCAAT-TATAgAA (positions 12013–12047, forward primer carrying a mismatch indicated by the lower case g); Rev, TTC-CCACCCCTTCTCAGCCAATG (positions 12 164–12 186).

This PCR-generated mutation creates a recognition site for EcoRI that is disrupted in the LA9[2002] cell line (12049C). The restriction fragments generated were analyzed using 5% polyacrylamide gel electrophoresis.

Allele-specific termination of primer extension analysis

To determine the length of the A track at positions 5172–5182, allele-specific termination of primer extension was carried out. Thus, a 344 bp fragment containing the O_L region was amplified by PCR with the following primers: (i) Fw, CAAACAAAAACTAAACCCAACC (positions 4862–4883); (ii) Rev, CTCTACTAAGACTTCTACCGCC (positions 5184–5205).

The PCR-amplified fragments were used as templates, and the 5′ end ^{32}P-labeled OL-PE oligodeoxynucleotide was used

as a primer: OL-PE, CTTCAATCTACTTCTACCGCCG (positions 5150–5171). Nucleotide concentrations were 50 μM dATP and 500 μM ddTTP.

Primer extension was also used to verify the length of a track of A residues starting at position 9821 in the *tRNA^Arg* gene. Thus, a 377–379 bp fragment containing this region was amplified by PCR with the following primers: (i) Fw, CTACTTCCACTACCATGAGC (positions 9672–9691); (ii) Rev, GTATGGAGCTTATGGAGTTGG (positions 10 028–10 048).

In this case, the 5′ end ^{32}P-labeled Arg-PE oligodeoxy-nucleotide was used as primer: Arg-PE, GGATTAGAAT-GAACAGAGTAAATGGTAATTAGTTT (positions 9786–9820).

The primer-extended products generated were analyzed using 8% polyacrylamide/7 M urea sequencing gels, dried and exposed for autoradiography.

RESULTS

The comparison of the mtDNAs from the different mouse strains reveals that only the sequences of NZB/B1NJ and Mil P mtDNA are very divergent from the LA9[1981] mtDNA (iden-tities = 16 197 and 12 gaps for NZB/B1NJ; identities = 16 202 and 12 gaps for Mil P) and from each other (identities = 16 236 and no gaps). On the contrary, the mtDNA sequence of all the other mice strains investigated turned out to be quite similar to each other and to LA9[1981] mtDNA (Table 1). Interestingly, in the case of the inbred strains, the predicted evolutionary relationships between these mtDNAs (data not shown), agree very well with their genealogy (6). This observation strongly suggests a recent common female ancestor for all the inbred strains investigated, except for the NZB/B1NJ, regardless if they belong to groups A (Swiss mice), B (Castle's mice) or E (C57-related strains) (6).

As shown in Table 1, the C3H/He, Balb/cJ, A/J and NOD/LtJ inbred strains derived mtDNAs are identical in all but one locus, at the *tRNA^Arg* gene (position 9818 in the LA9[1981] sequence and position 9821 in the remaining inbred mice sequences). This difference is mainly due to the variation in the length of a stretch of As in the *tRNA^Arg* DHU-loop, which seems to be a highly polymorphic locus in mice (7). Besides this locus, other mouse strains differ from the former group in only one (AKR/J and SKH2/J), two (SAMR1 and C57BL/6J), four (SAMP1) or five (SAMP8) additional bases. Remarkably, the mtDNA non-coding regions (also called *D-loops*) of all these mouse strains are identical, while they differ from the genetically distant mouse strains NZB/B1NJ and Mil P (eight mismatches and one nucleotide gap with the former and nine mismatches and one nucleotide gap with Mil P, whereas between NZB/B1NJ and Mil P there are seven mismatches with no gaps). The observation of changes in the coding sequences between the related mouse strains, while the mtDNA non-coding region is strictly conserved, is in sharp contrast with what has been described in human mtDNA (11 and references therein).

It should be mentioned that the number of nucleotides in the related mouse inbred strains ranges from 16 299 to 16 301 bp, the differences being due to changes in the length of the stretch of As in the *tRNA^Arg* DHU-loop. In addition, in the unrelated strains NZB/B1NJ and Mil P, there are insertions of a base in

the tRNA^{Cys} T-loop (data not shown) and one nucleotide in the mtDNA non-coding region (D-loop) that enlarge the size of the sequence to 16 303 bp.

The comparison of the mouse inbred mtDNA sequences with LA9^{1981} reveals differences in 15 loci: eight in open reading frames (ORFs), four in tRNA or rRNA genes, and only three in the D-loop. Remarkably, only one of the substitutions within the ORFs is silent, the remainder provoking the insertion of a full codon in the reading frame of two protein-coding genes (Nd3 and Nd4L) and the substitution of one amino acid in five loci (Table 2). The fact that LA9^{1981} lacks two full codons, and has only eight As in the polymorphic locus at the tRNA^{Arg} DHU-loop explains the shorter size of its mtDNA (16 295 bp). The size variation in the mtDNA of the different sources makes the description of their comparison complicated. Thus, to avoid confusion we have chosen to give the actual numbering of the sequence that we are commenting on followed, when different, by the corresponding number of the LA9^{1981} in brackets.

In principle, the differences between LA9^{1981} and the inbred mice strains could suggest a different genetic origin for the LA9 mtDNA and the rest of the inbred strains described to date, as seems to be the case with the NZB/B1NJ mtDNA. This interpretation could be reinforced by the presence of differences in the mtDNA D-loop sequence. Intriguingly, however, all but one of the different loci in the D-loop region, are also conserved between the mtDNA of the distant strains NZB/B1NJ and Mil P. This raised the possibility that the differences between LA9^{1981} and the mouse-derived sequences could be due either to acquired polymorphisms during the lifetime of the cell culture or to sequencing errors in the earlier sequencing.

To distinguish between these two possibilities we have performed the full sequencing of the LA9 cell line mtDNA (called hereafter LA9^{2002}) and an additional L-derived cell clone (L929) mtDNA. The L929 cell clone (ATCC CCL-1) was established in 1948 by the capillary technique for single cell isolation, it being the first cell line derived from the parental L strain (12). Microsatellite analysis has allowed us to discriminate the two L-clones (LA9 and L929) from C57BL/6J, C3H/He, Balb/cJ and CBA/J, but not between them, supporting their genetic relationship (data not shown). The similarity between the mtDNAs of LA9^{2002}, L929, Balb/cJ, C57BL/6J and the rest of the inbred strains indicates that most of the problematic loci detected in the reference sequence were very likely due to sequencing errors (Table 1). However, we found at two loci the same nucleotide in LA9^{2002} as that reported in LA9^{1981}, being discrepant with that shown in the L929 and the mouse inbred mtDNAs [positions 4794 within Nd2 and 12 049 (12 042) within Nd5]. Moreover, L929 mtDNA also showed some loci [positions 6063 and 6589 within CoI and the insertion of a C at position 13 887 (13 879) in Nd6] that differed from LA9^{1981}, LA9^{2002} and the rest of the known mouse mtDNA sequences (Table 1). In addition, we could not resolve the discrepancy between L929 and LA9^{1981} in the length of the stretch of As in the tRNA^{Arg} DHU-loop, since the LA9^{2002} sequence (nine As) differs from both L929 (10 As) and LA9^{1981} (eight As). To fully guarantee the accuracy of the obtained sequence information and to establish the heteroplasmic or homoplasmic nature of the observed variations, all the discrepant positions were confirmed by

Table 2. Coded amino acid of the sequence variants described for mtDNA[a]

Nucleotide position	Protein	Amino acid position	Cell lines		Mouse strains												Proposed reference
			LA9^{1981}	L929	LA9^{2002}	C3H/He	AKR/J	Balb/cJ	A/J	NOD/LtJ	SKH2/J	SAMR1	SAMP1	SAMP8	NZB/B1NJ	Mil P	C57BL/6J
4012e	ND2	33	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu
4794b	ND2	294	Ile	Thr	Ile	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr
5734e	COI	136	Pro	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu
5737e	COI	137	Val	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala
6063c	COI	246	Leu	Leu/Ile	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu
6238e	COI	304	Cys	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr
6589c	COI	421	Val	Ala	Val	Val	Val	Val	Val	Val	Val	Val	Val	Val	Val	Val	Val
9348	COIII	248	Ile	Val	Ile	Ile	Val	Ile	Ile	Ile	Val	Val	Val	Val	Val	Val	Val
9461e	ND3	1	Met	Met	Met	Met	Met	Met	Met	Met	Met	Met	Met	Met	Met	Met	Met
9554e	ND3	33	–	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
10 059e	ND4L	64	–	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile
10 841	ND4	227	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly
11 175	ND4	339	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Pro	Pro	Ser	Ser	Ser
12 042b	ND5	103	Leu	Phe	Leu	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe
13 046	ND5	437	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe
13 879c	ND6	42	Gly	Gly/ frameshift	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly

[a]The table shows differences in the mtDNA sequence at the indicated loci, the remaining positions are identical between all the sequences with the exception of the two more divergent ones (NZB and Mil P). The positions at which the LA9^{1981} sequence is likely to be wrong are followed by an e.
[b]In culture, mutations acquired by LA9.
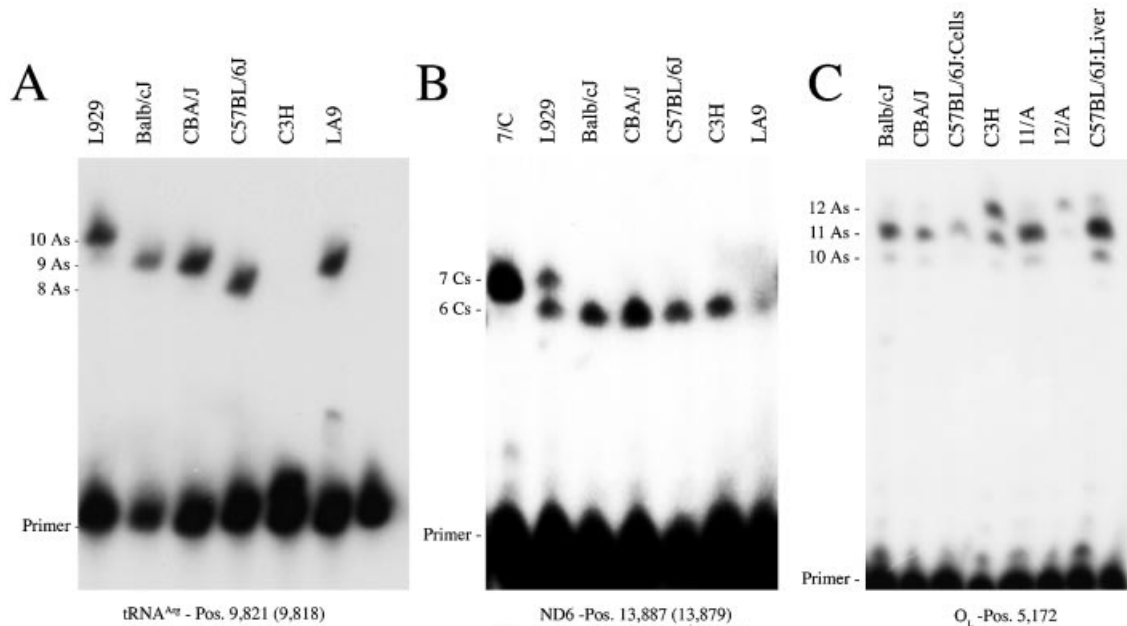[c]In culture, mutations acquired by L929.

Figure 2. Allele-specific termination of primer extension assays designed to establish the size of three polymorphic mononucleotide tracks observed in the *tRNA*[Arg] gene (**A**), the *Nd6* gene (**B**) and the L-strand replication origin (**C**). Note that the extension of the C3H mtDNA sample in (A) cannot be longer than 1 nt due to its particular four Ts + nine As sequence, while the rest of the samples harbor three Ts + *n* As, with *n* = eight, nine or 10. Lines without label in (A) and (C) represent un-extended primer. Lines labeled 7/C, 11/A or 12/A in (B) or (C) represent controls for seven Cs or 11 or 12 As in the respective polymorphic track.

RFLP analysis (Fig. 1) or allele-specific primer extension termination (Fig. 2). We detected heteroplasmic loci in the L929 mtDNA (position 6063 in *CoI*), it being 18% C_{wt} and 82% A_{mt} (Fig. 1B). In addition, the L929 length polymorphism due to the insertion of a C at position 13 887 (13 879) in the *Nd6* gene was found to be heteroplasmic, it being present in 52% of the mtDNA molecules (Fig. 2B). The additional private polymorphisms in L929 (position 6589 within *CoI*) or LA9 [position 4794 in *Nd2* and position 12 049 (12 042) within *Nd5*] were homoplasmic within the limit of resolution of the RFLP analysis (Fig. 1A, C and F). All these variations would represent clone-specific mutations acquired in culture (10,13).

RFLP analysis confirmed that the two C57BL/6J-specific polymorphisms were also homoplasmic (Fig. 1D and E). Interestingly, we could not detect any heteroplasmic loci in the sequence of the Balb/cJ mtDNA, but we found a length track polymorphic locus within the sequence of C57BL/6J from the Sanger Institute, consisting of a stretch of either 11 or 12 As (starting position 5172) in a short non-coding segment proposed to contain the replication origin of the mtDNA L-strand (14). Allele-specific primer extension termination (Fig. 2C) demonstrated that this locus is homoplasmic in a DNA sample from a mtDNA-less cell line repopulated with either mtDNA from C57BL/6J, Balb/cJ or CBA/J mouse platelets (10,15). However, this locus is heteroplasmic for 11 and 12 As in a DNA sample isolated from C57BL/6J mouse liver and in C3H/He mouse-derived cells (Fig. 2C). Notice that the termination signal at 10 As in this figure is due to a nuclear pseudogene harboring 10 As (unpublished results). Similar heteroplasmic length variants in non-coding regions of the mtDNA from a single individual have also been

documented in humans (16). Finally, the combination of sequencing analysis and allele-specific primer extension termination, allowed us to confirm the existence of all the variations proposed for the polymorphic locus at the *tRNA*[Arg] DHU-loop: the length variations in the stretch of As (Figs 2A and 3), as well as the particular sequence of C3H/He-derived cells consisting of nine As plus one T (Fig. 3).

It should be remarked that despite the number of confirmed errors (10), the sequencing effort performed by Bibb and co-workers in the earlier 1980s, provided a very high quality sequence with only a 0.08% error frequency.

DISCUSSION

Based on the overall data, we consider that both L929 and LA9 mtDNAs showed fixed and heteroplasmic polymorphisms acquired during their lifetime as independent clones, that were very likely not present in the mouse from which L cells were established. Worryingly enough, we have found evidence proving that the L929 clone-specific homoplasmic and heteroplasmic mutations have phenotypic consequences for the proper function of the mitochondrial respiratory chain (10,15), and this may also be the case for the LA9-specific mutations. Special mention should be made of the polymorphic locus in *tRNA*[Arg]. This gene presents a stretch of eight As (LA9[1981], SAMR1, SAMP1, SAMP8 and C57BL/6J mtDNAs), nine As (LA9[2002], Balb/cJ, NOD/LtJ, SKH2/J and AKR/J), nine As plus one T (C3H/He) or 10 As (L929, A/J, Mil P and NZB/B1NJ mtDNAs). Interestingly, it has been recently reported that the length of this stretch of As can modulate the pathological expression of a nuclear locus involved in aging-related deafness in mice (7).
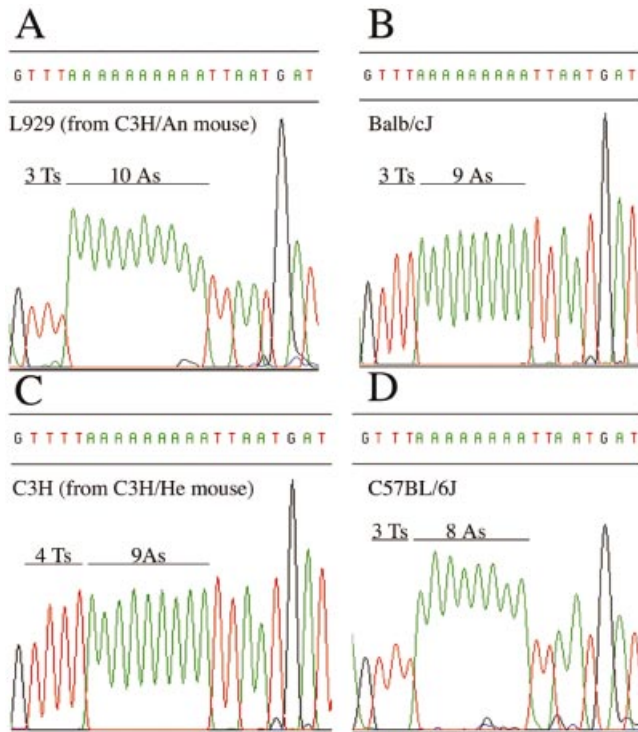
Figure 3. Representative chromatograms that illustrate the variety of viable sequences described to date in the highly polymorphic locus at the mouse mtDNA-encoded *tRNA^Arg* gene. The source of the DNA is indicated in each panel.

According to the data presented here, the reference sequence for mouse mtDNA should be reconsidered for the following reasons. (i) It contains seven substitution errors, three single nucleotide insertions and a single base and two triplet deletions. (ii) The two clone-restricted polymorphic positions in LA9 do not represent the wild-type version present in the original C3H/An mouse and they could likely be pathogenic. (iii) Since LA9[1981], LA9[2002] and L929 still diverge in the polymorphic locus at *tRNA^Arg*, a highly variable locus in mtDNAs of mouse strains, we cannot predict the more likely sequence for this locus in the original C3H/An mouse.

Therefore, we propose the sequence of C57BL/6J mtDNA as the mouse reference in order to have a true reference sequence and not a consensus one. In that way there will also be a match between the reference sequence for the nuclear and the mitochondrial genomes in mouse. Note that if these recommendations are followed, the size of the mouse mtDNA reference sequence will increase from 16 295 to 16 299 and the nucleotide numbering should be modified accordingly.

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