Efficient Cloning of Entire Mitochondrial Genomes in *Escherichia coli* by *In Vitro* Insertion of a Transposon

Young Geol Yoon and Michael D. Koob  
Institute of Human Genetics, University of Minnesota, Minneapolis, MN

**Introduction**

The typical animal cell contains hundreds of mitochondria that produce the cell’s ATP through oxidative phosphorylation and regulate multiple cellular processes. Even though the majority of genes needed for the biogenesis, maintenance, and regulation of this organelle are encoded in the nucleus, the mitochondrial genome is critical for normal cellular energy metabolism, and mutations in this DNA molecule are known to cause a wide range of human diseases. Generally, mammalian mitochondrial genomes consist of circular double-stranded DNA (~16-17 kb) that encodes 13 polypeptide subunits of the mitochondrial ATP-generating pathway, two rRNAs, and 22 tRNAs. We have devised an efficient method for replicating and maintaining entire mitochondrial genomes in *E. coli* and demonstrate the effectiveness of this procedure by cloning individual mouse mitochondrial genomes isolated from mouse liver. The same approach has been used to clone or “rescue” plasmid DNA that would not otherwise replicate in *E. coli*.2,3

**Materials and Methods**

The strategy for the EZ::TN™ transposon-mediated cloning of mitochondrial genomes is shown in Figure 1. A transposon, in which the R6Kγ origin of replication (R6Kγori) and a chloramphenicol resistant gene (CmR) are flanked by mosaic end (ME) sequences, was generated by a PCR reaction using a R6Kγori/CmR-carrying plasmid as template and the primers MESalCmR (5’-CTG TCT CTT ATA CAC ATC TGT CGA CAG AAG CCA CTG GAG CA-3’; ME sequence italicized, Sal I restriction site underlined) and MESmako (5’-CTG TCT CTT ATA CAC ATC TCC CGG CCT TAT TCT GTC AGC CGT T-3’; Sma I restriction site underlined). The in *vitro* transposon insertion reaction consisted of 1 Unit of EZ::TN™ Transposase (EPICENTRE), 200 ng of mouse mitochondrial DNA (mtDNA), and 10 ng of PCR-amplified transposon in the buffer provided by the supplier. After incubating the reaction mixture for 2 hours at 37°C, the reaction was terminated as described in the product literature. A 1-µl aliquot of the transposon insertion reaction was used for electroporation of an *E. coli* strain containing the wild-type pir gene (pir+).4 Transformants were selected on LB plates containing chloramphenicol (12.5 µg/ml).

**Results and Discussion**

**Cloning the mouse mitochondrial genome in *E. coli***

For cloning complete individual mitochondrial genomes in *E. coli*, we devised a scheme that uses an *in vitro* transposition reaction3 to insert an *E. coli* origin of DNA replication and selectable marker at random locations into circular mitochondrial DNA (Figure 1). The 1.5-kb PCR-amplified transposon consisted of a CmR marker and a R6Kγori flanked by the 19-bp inverted ME sequences that are specifically recognized by EZ::TN Transposase. To perform the *in vitro* transposition reaction, this linear transposon was incubated with EZ::TN Transposase and purified, circular mouse mtDNA. The products from this *in vitro* transposition reaction were electroporated into an *E. coli* strain containing a chromosomal copy of the R6Kγ pir gene and transformants were selected on chloramphenicol plates. The pir gene encodes the replication-initiator protein π needed for R6Kγori replication.

We characterized three transposon-inserted mouse mitochondrial genome clones obtained from this cloning strategy by restriction enzyme mapping and sequencing (Figure 2). This cloning procedure takes advantage of the fact that the mitochondrial genome is the only circular DNA in most eukaryotic cells and that DNA must be circular in order to replicate in *E. coli*. Since the transposition reaction does not circularize linear DNA, the genomic DNA fragments and broken mitochondrial genome fragments that invariably contaminate mitochondrial genome preparations are not cloned by this procedure and so the background was very low. The replication ori and selectable marker were also inserted at random locations and orientations throughout the mitochondrial genome and so many different clones were generated in the same experiment. All of the transposition reactions that generated the mouse mitochondrial genome clones described here inserted the transposon in the same orientation with respect to the mitochondrial genome and this probably reflects an inherent increased stability for this orientation versus the other. The transposon cloning approach we describe here can be used to clone completely...
uncharacterized, circular mitochondrial genomes with no prior knowledge of either the sequence content or restriction pattern of that mitochondrial genome.

Comparison of the stability of mouse mitochondrial genome clones in E. coli at low- and high-copy number

To examine the stability of the cloned mouse mtDNA in E. coli, the three mapped mouse mtDNA clones were transformed into one of two E. coli strains. One of these strains contained the wild type pir gene (pir+) in the chromosome and replicated the mtDNA clones at a moderately low number of copies/cell (10-15 copies), and the other strain contained a mutant pir gene (pir-116) that replicated the clones at a relatively high number of copies/cell (~200 copies).4 As shown in Figure 3, lanes 2-4, no deletions or rearrangements were observed in the mouse mtDNA fragments when we used the pir+ strain as host. When the mouse mtDNA clones were transformed into the pir-116 strain, however, the transformation efficiency was dramatically lower than that seen with the pir+ strain, whereas a control plasmid without mitochondrial sequences was transformed into these two strains with equal efficiency (data not shown). Restriction analysis of the mtDNA clones from the pir-116 strain demonstrated that all or most of the mouse mitochondrial genome sequence was deleted (Figure 3, lanes 5-11). The clone instability we observed in high-copy clones was probably due to the inhibition of E. coli cell metabolism by one or more of the 22 heterologous tRNA genes in the mouse mitochondrial genome. We identified multiple regions of the mouse mitochondrial genome that serve as transcription promoters in E. coli and found that the level of transcription from these sequences was dramatically higher at high-copy numbers than at low-copy numbers.6 By using a pir+ strain to keep the clone copy number low, however, the level of expression of the tRNA genes or other potential mtDNA inhibitory sequences was minimized and the overall mitochondrial clone stability was increased.

Conclusion

We have cloned the mouse mitochondrial genome in E. coli by inserting an E. coli replication origin and selectable marker into mitochondrial DNA using an in vitro transposition reaction and then transforming the modified genomes into E. coli. The entire mouse mitochondrial genome was very stably maintained when its copy number was kept low (pir+ strain), but was extremely unstable when it was replicated at high-copy number (pir-116 strain). This convenient approach should offer a number of advantages for the cloning of other mammalian mitochondrial genomes in E. coli.

References


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