IMPORTATION OF MITOCHONDRIAL PROTEIN BY AN ENHANCED ALLOTOPIC APPROACH

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ABSTRACT

An expression vector containing appropriate mitochondrion-targeting sequences (MTS) and appropriate 3'UTR sequences provides efficient and stable delivery of a mRNA encoding a protein (CDS) to the mitochondrion of a mammalian cell. The MTS and 3'UTR sequences guide the CDS mRNA from the nuclear compartment of the cell to mitochondrion-bound polysomes, where the CDS is translated. This provides an efficient translocation of a mature functional protein into the mitochondria. A method of targeting mRNA expressed in the nuclear compartment of a mammalian cell to the mitochondrion is also provided. The vector and methods can be used to treat defects in mitochondrial function.
FIG. 1B (Cont'd)
SOD2MTS- mATPG- SOD2 3' UTR (SEQ ID NO:22)

GAATTCCCTTCTGCTTAGATGTGAGCCGGGACAGTGCGGCCACCGAGCGACGCTGCTTCTGGCTTGGG
TATCTGGGCTCCAGCGAAGACAGCAAGCTCCGCAGCCGCTTGACGATGAAGAGAATTCGTTGGGCTC
ATTGCCCGACAAATCTTGGCGCCGCGAGATCGATGATATCTATTTCTCCCTCTATTGATCCCGACCTCC
AAATATGCTTCAGACACCGTCTACCCCGACCGACGACGCTGCTACGATCCTCAACTACCCATCAAAAG
ATACCACTAAAGAAGACGGAACCACTGGCTTCATATCTATGATAATTCTTTTAGCCACCAGACTAACCTC
CTGCTAGCTGCTTGCTTACATGCTATGAAAGACCGACCCACACACTACATAGAACCTGCCCGCCAGCGC
CAGCCCGATCATGCTTCTTCTATGAAAGACCGACCCACACACTACATAGAACCTGCCCGCCAGCGC
CTAGCTAGCTAGCAGCTTCTTCTATGAAAGACCGACCCACACACTACATAGAACCTGCCCGCCAGCGC
CAAGCTAGCTTCTTCTATGAAAGACCGACCCACACACTACATAGAACCTGCCCGCCAGCGC

FIG. 1B (end)
**FIGURE 2**

- **COX6c**
  - 250 nt

- **MTS COX10-ATP6**
  - 780 nt

- **ATP6-3' UTR COX10**
  - 2374 nt
Anti-Flag M2

Anti-ATP synthase β

pCMV-Tag 4A vector

MTS COX10-nATP6

MTS COX10-nATP6-COX10 3'UTR

FIGURE 3
FIGURE 5A
FIGURE 5B (Start)
COX10 MTS-nND1- COX10 3' UTR (SEQ ID NO:28)

ACAGGAGATTACAAACAGTACGCGATAGCTAGGCCGCGGTATCTTATTATATAGGTACACAGGCTAGCTGCACT

FIGURE 5B (Cont'd)
FIGURE 5B (end)
nATP6 - SEQ ID NO:27
ATGACCAGAAAAATCTGCTCGCTTTCAATTCATGCCCACAATCTCTAGGCCTACCC
GCCGACTAGTACTGACTATTCTATTCTCCCGCTCTATTTGTCCACCCACCTCAATTC
TCATCAACAAACGACTAAATCACACCCACACACAAATGCTAATACACTAACACTAAA
ACAAATGATCAGGTACAGGTGAACACAAGCTTAAAGGACGAACTTGTCTCTATGCTAGT
ATCCCATTATCTTTTATGCGCACTACACTACCTCTGCTGGACTGCTCCTACCTCA
TTTACACCACCCACACACTATCTTGCGACTGCTACGCTCCCTATTTATTGAGAAGT
CCATCGGTACACCTACATTCAACCAATGGGCCTGCGCCTAGCGCTAAACGCCCTAC
TTACTGACAGGCCCACCTACCTACATGCACTATTTGGAAGCCACACCTGACTGAT
CAACACCATATACTTCTCCTACACTATCTTCTGACAAATCTAATATTACTGACT
ATCCTAGAAATCCGGTACGTGCCAATCTCAAGCTCCACTTTTACAACTTCTAGTAA
GCCTACTACCTCAGCAACACAGCGGCGCAGCGAAGGCC

nND1- SEQ ID NO:28
ATGCCAATGGCAAAATCTGCTCGCTCTCATTTCATGCCCACAATCTCTAGGCGCCACT
TTCCCAATGCTGACTGAAAGAAATTTCTGGATACATCGAGCTCAGGAAGGGG
CTAATCTCTGGGTGCTACCTCATCTGCTGTCTGCGGACTGCTTGTGTGATGAGG
CTGTTTCAAAAGAGCGCCCTGGCAAACCCAGGCACCTTCAATCAACCCGCTGCTATT
ACCGCTCTCTCCCGCTGACAAATCTGCGCTGGCTTCTTTCTATCTGCGGACCC
AGACGCTCTGGCCTGTTACTCCATTCTGTGGAGGCTAGGGTCTTTGCAAGCAGAA
TTACGCCCTGATGCGTGTGCGCTGAGGCCTTGGCGCAGCACATTCTTGACAGG
TGACCTCGCCACATTCTCGCTCTCAACCTCTGCTGATGAGCGCTTTCTACAC
TCTCAACCTGATTACAAACGAGGACACCTCTGGCGTCGTCCTCGCCAGCTGCGG
CAGCTGCGCATGATGGTTTATCAGCACCCCTGCTGGAGACAAACGGAGCCCCC
TTCTATGCTGCGCTGGAGGCTAGGCTGTGGTCGCTGGAGCTCCGACTTAATATTAGG
GCAGAGGGCGCTTGCTCTTCTCTATGCAGGCGTATACAAATATTTATTGAG
TGAAACACATGTCACTACTATCTGTCTCCTGGTACATAGCAGCTCTGAGGG
CGAATCTGCTGAGGCTGAGGCTGTCGCTGGAGCTCCGACTTAATATTAGG
GCAGAGGGCGCTTGCTCTTCTCTATGCAGGCGTATACAAATATTTATTGAG
TGAAACACATGTCACTACTATCTGTCTCCTGGTACATAGCAGCTCTGAGGG
rND4- SEQ ID NO:29

ATGCTGAAGCTGATGCTGACACCATTATGCTGCTGGCCTCTGACATGGCTGTCT
AAGAAGCCATGATCTGGATTAAACACAAACCACCCACGCTGATTCTCCATCA
TCTCCCTCTTGTTCTTCAACACAGATCAAACAACCTGTTTCTCTGCTCACCTAGC
TTTTAGCAAGCAGGATTCACTGACAACCCCCACACTGCTGATGCTGACAACCTGGCTCCT
CCCTGACATACTGATGCTGCTGCTATTTTCTCCAGATCTCTACATCACTGAC
CTTCACTGCCAGGCTATGATTTATGTCCTATATTTTCTCTGAGACTACTACTGTAC
CCTACATCAGGCTATTACACCCGGTGCGGCAACAGCCTGAGAGACATGAC
CGGAGCTATTATTGTCTTCAACACCTGGTGGGTGCTAGCTGGCCCTGCTGATGAC
CGGTCTGACTACACCATAAAACACTGCTGCTTCATGATCCTGTGACCTCAC
QACAGGCCAGGAGCTGTCACATCTCTTGGCTACAACATCATGTGTGGCTGCTGACATA
CAGTGAGGCTTCTACATGGTAAGACTGATCCTATATTTGACTGTCACCTCCTGGCTCCT
TAAGCGCCACTGAGGACCACCATTTGAGGTGCTACGTGCTGGGAGACTGTCG
TCCTAGAAGCTGGTGCTATGGGATGATGGCCTGACCCCTGATCGATCGAAATCCT
CTCAGCAAGCATTGCTATTACCTTGTGTGGCTGTGGTCCTCTGTTGGGGAAATGATT
ATGACAAGCTCATATTGTCTGGCAGACAGACAGCTGAAAGCTGATTTGCCTAC
AGCATGATTACATGATGCTGGCTGCTGCTGACCCTATTCTGAGATCAAGACACC
TGGGTCTTTTACAGGGCTGCTTATGATCGACGCCAGACTGACCTCATCA
CTCCTGGCTCTGTCTGGGCCACTCAACTACGAAAGGACACACTCAAGAATTTAGA
TTCTGAGGCTGGACTCGAGCTCTCCTGCTCCCTCCTATGCTCTGTGGCGTCTG
CTCAGCCTCTCTGAGGCTATTGCCTGACCCCTGCTTCTCCAAATACATCTGTTGGGGGA
GCTAGGCTGCTGTCAGACCTTTTATTGTTGTCACACATCAGTCTGTTCACCTACACAC
ACAGTGCGGGTACCCACTCATTACATAACATGAAAGCCATTCTTCTTTACTAGG
GAAAAACTCTGATGTTTATGCTTCTGCTCCCAATCTCCTTCTGACTGATGTTGACCC
CCGACATCATGACCCGGCTTTAGCTCT
COX10 MTS- SEQ ID NO: 30
ATGGCCGCACTCCGGCACACATCCTCTCTCACGGAACCTCTGGACAGTTGCGTAGG
AGGCTCTGTGTGCTGTGATGCTGAGCCGCG

COX10 3'UTR- SEQ ID NO: 47
GAGCAGTCGGACCGCGCCACCCCGCCCGGCTCCCGGGCTGCCAGCAAGCAGCATGTTG
TGTTATTTCTGGAACACAAAGAAAGAATTGCTGGGTCTGAAGAAGTTTAATAAAA
CGAATTCGGATCCAGTCACTGAGCTGTACGTSGATGTTTGAGTAACAACTAAG
AAATATCCGCCCTTCTTTGAGGCGTTTTTATATCTCTCCTCAACCCGACCGCTATCC
GTTATTTTTCTCTCCACATGGGGAGAGACTCACTACAGCTGTCCCTATTGAGTCC
CTCTACCACACGACACACCCACGCACACTCCACATGCCGAGAGTTGGCACTTTG
GTGCGCCAGAAAGTGGTGAAGCTCAGTGCAGTGTCTGCTGCTGCTGTGCTGTGCTGTGCTG
GTCTCAGAAAGCCCGCCCTCTCTGCTCTCGTCAGCGAGCTCGAGGATTTCCACATTTTGCC
TTGGAGAGTTCTGCAAGTGGCTCGCCAGGCGCCTGTCTCCTCCTCCTACCCGCTTGG
GTAGGACATTTCCAGAATCCTCAAGGAGTCAAGGCACTTTTATAGTTTCACGTATTAA
CATATAGAGACGTGGAAAGCAGCTTCTCTCTTTAAAGGTTAGCCCTGGAGCTAAATAT
CCACGGGATACCTCTGGGCCCCACCACCCATATTACTAGCCTCTGGAGCTACTACT
GTGGGGCGCTCCACTCTGCTAGACACAGCGGCTTTTGGAGGTATGGGAGAGTGGAAG
AGGGAAGTTAGGAAGAAAGGTGCTGGTCTGGCAAAACAGGAGCCAGCAAGCTCAGAT
TCCTGCTCTTTGCTGAAATAATACATGCTCACTCTGTGTATCTCCTGGAGTTCCAGA
ATTAGCTCCCAATGTCGAAATGGCTTTTAAAGGGCAGCAAGAGGAGGTGGTCTGGGAT
TTTGGAAGTTATCTCTGGCCAGGTGCTGGTCTGGTCTGGTCTGGTACACAAATACGCGATTACCT
GCCAGCTTTTTAGTCCTCTGCTCCACGGGCTGCGAGCTGCCACATCTGGCCCAA
AGGGTGCTAAGGGTCCACGGTGTAAAAATTAACAGCTGTCATGGGAGGCTGGAAGAAG
GGTTACTCAAAATGTCCTAAAGGAAGTGTGAGTTGAGTGAATACACATCCAAATACCTGTT
GCCAACTTATGCTTTAATCGGCCCTGTTGGCCTCCGAGATTTAGTGGGAGAA
CATGGGATAGGAATGTCCTGAGACGAGCTCAGTCCAAGGAGCTTCATTAGCAGGCTTACCAT
TGATAGAAGCTTT

SOD2 MTS- SEQ ID NO: 31
ATGGTGAACGGCGAGGTGTCGGACACCAGCACAGCAGTCGGCTCCGGTTTTGG
GGTATCTGGGCTCCAGCAAGACAGCACAGCCCTCCGACGCCTGCGACGCG

SOD2 3'UTR- SEQ ID NO: 35
TTATGCTGAGTTAGTTAAGCTCCTTTTAGACTGTTTTTGTAGTGGGATAGGACTTG
CAGAATACAGTGAATTTATGATGCTTATGGAATGTTACATGCTGCTGCTGGGAGTCTTATGCATT
TTTCTAAGAACACTTAATGGGCTGAATACCTAGTTACCTCTAAACATTATTTGGTGTTATTGGGC
AAGTGATGAGCAAATAGTAAATGGCTTTGCTGGATT

**FIGURE 10**
Aconitase (ACO2)
Accession number: NP_001089

Protein Sequence: 780 aa (SEQ ID NO: 48)
mapyslitr lqkalgvyrg hvaslvcqra kvamshfepn eyihydiek ninivrkrin
rpltskiv yghildpasq ierengksylr lrpdrvamqd ataqmamliq issglskvav
psyhodhli eaqyggekdi rakdinqeq yenflataqg yyygyfwkgps glihqlilen
yayqygifv tdshtpnggg llggicigvgg adavdvmagi pweikcpxv gyklqglsig
wsspckvilk vaglftvkgk gaiweyhgpf gydsistcgm aticnmgaei gattsvypyn
hrmkylsksk gredianлад edfklhvdpdp gchydqliey niselkphin gpfipdlahehp
vaevgykvaek egwplidvrg ligscsnsy edmgrsaava kqalahgkic ksqfltgpfs
eqiratierd gyaqilidlq givlanacgp cizqwdikdi kkeqntitv symnftgm
danpethahf vseivtala iagtkhpe tydylgtest kfrleapdad elpkgefdppg
qdyhqppkd ssgquhtvdsv tspqfllep fdkwdgdkle dlqlllkgk kcttdhisasa
gpwlkfrgfl dmnsnnllq ainiengkan svravvaqef gwpdtaryy kkhgirwvvi
gdenyeggs rehaalephr lgrsaliks farihenthik kaglpltfapadynkikh
vdkltiqglk dfpfpilklc 1ikhpngtqeq tillntftne tglewfrags alnmkelqy

MTS sequence
Net charge of query sequence: +0
Analyzed region: 37
Number of basic residues in targeting sequence: 5
Number of acidic residues in targeting sequence: 0
Cleavage site: 31
Cleaved sequence: MAPYSLLVTRLKQALGVRQYHVASVLQRA (SEQ ID NO: 32)

3'UTR sequence (Accession # CC373828; SEQ ID NO: 33)
gggcagttgcc ctggcggccc gggcgcqgttcg gcggcggttc gcggcgggttc gtcggcgttcg 60
gggcgacttc gccggcgttc ggggttgcttc gcggcggttc gcggcggttc gcggcggttc 120
gccgcgcgt ctagccggct gcggcggttc gcggcggttc gcggcggttc gcggcggttc 180
tttccggccc ggtccggatc cgggttggatc gcggcggttc gcggcggttc gcggcggttc 240
tttttttttt ggcgtcttcc gccggccatc cgggttggatc gcggcggttc gcggcggttc 300
gttttggttgg gggcgggttc gcggcggttc gcggcggttc gcggcggttc gcggcggttc 360
aggggggggg ggcccccccc ccgggggggg gcggcggttc gcggcggttc gcggcggttc 382

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FIGURE 11 A
**Superoxide dismutase mitochondrial (SOD2)**

Accession number: **P04179**

Protein Sequence: 222 aa (SEQ ID NO: 49)
mlsravcgt srlapalgyl gsrqkhslpd lpydygalep hinaqimqlh hskhhaayvn
niitvekyq ealakgdvta qjalapafk rggginhnsi fwnlspngg gepkgellea
ikrdfsfdk fkektaasv gvaqsgwgwl gfnkerghlq iaacpnqjpl qqtglpl
gidwhehavy lqkyknvpyd ikaiwnvinw envterymac kk

**MTS sequence**

Net charge of query sequence: **+2**

Analyzed region: **29**

Number of basic residues in targeting sequence: **4**

Number of acidic residues in targeting sequence: **0**

Cleavage site: **21**

Cleaved sequence: **MLSRAVCGRSRLAPALGYL** (SEQ ID NO: 34)

**3'UTR sequence (Accession # CC263966; SEQ ID NO: 35)**

```
accacgatcg ttagcctgat ccaaccctca tgcctgccgc aagataacgt ctcgctttct  60
aagatacga toaaacctgy ycaatactgya aaccctata aagttcctgga taactttgtg 120
tgtttata attggaaaga caatttattt ccacatgtgt gcagtitttt acctgttata 180
aaccacctg tcaaccctca mmmmmmma saaa 214
```

**FIGURE 11 B**
ATP synthase subunit beta (ATP5b)
Accession number: NP_001677

Protein sequence: 529 aa (SEQ ID NO: 50)
mlgfvgrvaa apasgairf tspaslpaq llraaptav hpyrdyaqt spspkagaat
grivavigav vdqvfgdegl pilnalevq ghlgestvrt iamdglteigv
rgqkvlidsga pikipvgep lgrimmvige pidergpklt kqfapihaca pefmmsveq
eilvgiekkv dilapaykkg klgfmgagv gktvimeeli nrvakahgy svfagyger
tregndlyhem iesgvnikl atskvalvvg qmmepgara rvaitglvta eyfrdqaegq
vltifdnfir ftnqgesvsra ligritosav yoplatdmg tmgnerittt kgsitsvqai
yvpaaltdp apatfahid attvisraia elglipvdp idstsrimdp nivgehydv
argvciqsl yksliqdlai lgmdeledse kitvrsarqi qrlsqpfqv aevftghmgk
lvpketkg fqqjlageyd hlpeqafymv gpiieavaka dklaehss

MTS sequence
Net charge of query sequence: -15
Analyzed region: 71
Number of basic residues in targeting sequence: 7
Number of acidic residues in targeting sequence: 1
Cleavage site: 64
Cleave sequence: MLGFVGRVAAAAPASGALRLTPSASLPPAQLLLRAAPTAVHPYRDYAAQTSPSP
KAGATGRI (SEQ ID NO: 36)

3'UTR sequence (Accession # CC313884; SEQ ID NO: 37)
gggcttctgg tctcctgtaa tggccttct cttgccctta acccctaaaq cttctstartt 60
tctgtggtga tcgcasgqa ootgattgga agatatsttc tttcttgaata gtaatgaagty 120
ttttaaatg asatgtcacc eetccagaaaa aaaaaaaaaa aa 162

FIGURE 11 C
Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 (UQCRFS1)
Accession number: NP_005994

Protein sequence: 274 aa (SEQ ID NO: 51)
mlsvaarsgp fapvisatsr gvagalrplv qatvpatpeq pvldkprfl sreslsgqav
rplvasvglnvпасvсvsh dtkupdfse yrrelvdet kssressar kgfsylvtvgy
ttvgvyaak navaqtqfvsms sasadviaalak kieklslip egknmafkwr gkplfrhrt
qkeiegaeav elsqrdqdqhdldvkkpew villgvcthl gcypianagdg pgyyycpchg
shydasgrir lgpaplnlev ptyfetdddm vivg

MTS sequence
Net charge of query sequence: +3
Analyzed region: 38
Number of basic residues in targeting sequence: 3
Number of acidic residues in targeting sequence: 0
Cleavage site: 37
Cleaved sequence:
MLSVAAARSGPFAPVLSATSRVGAGALRPLVQATVPA (SEQ ID NO: 38)

3'UTR sequence (Accession # CC310484; SEQ ID NO: 38)
ggaactttga ctcaagtcat aggctcttt cagttttat gtcacocctag gaaacctttt 60
tgyagsggaag ctttctgctac tgaagttttc tttgaattat gagaagattg atgatgtatt 120
tgaacattc aaatgtgaact aaattgatt tttgttttgc taccctcagg cattctctts 180
atanagacac tgtttacact tgtttatgtc agtnaaaaaa anaaaananaa anaa

FIGURE 11 D
NADH-ubiquinone oxidoreductase 51 kDa subunit, mitochondrial precursor (Complex I-51KD) (CI-51KD) (NADH dehydrogenase flavoprotein 1); NDUFV1
Accession number: P49821

Protein Sequence: 464 aa (SEQ ID NO: 52)
matrrlglw slparsvrf sgdtapkk tsgslkdedr ifnlygrhd wrlkgslse dwyktkeili kqpdwilgei ktsgrirrgg agfptgkws fmnkpsdgrp kyivvnaeg egvtckdrei lrhdphklie gclyggramg araayiyrg efymeasnlq vairayeag ligknacgsg ydfdvfvrvg agayicgeet aliesiegkq gkprlkppfp advvgfcpt tvanvetvav spiticrgggt wfgafgfrern sgklfnisg hvnhpcvree emsvplkeli ekalaggyttg wdnllaipvgs gssptlpiks vctvldfd alvqaqtgll taaivmdrs tdvkaae lefykhesog qctporevgvd wmnkvmarvf rgdarpeao sioeskqic ghticalgdg aawpgvglir hfrpeleeerm qfaaqhqvaaqas

Séquence MTS
Net charge of query sequence: +5
Analyzed region: 36
Number of basic residues in targeting sequence: 7
Number of acidic residues in targeting sequence: 1
Cleavage site: 21
Cleaved sequence: MLATRLLLGSLSLPAVSRVF (SEQ ID NO: 40)

3'UTR sequence (Accession # CR041510; SEQ ID NO: 41)
ccccccccctttgctgtgtgtgctcctgtgtctgtccatgtgctatccccctgtattatgctgtcctaatccacatgggataacccgggaattaaatgccccgggcctttgctggcttccctcttgccccatagctttgtcttttgcttcctagctttgttttttggccttttggctttttgtttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt

**FIGURE 11 E**
**NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa: NDUFV2**

Accession number: NP_066552

Protein Sequence: 249 aa (SEQ ID NO: 53)

mffsaalar aagltahwgr hvrnlhtam qngaggalfv hrdtpennpd tpfdftpeny kriealvknk peghkaavf pvidlaqrgp gwipsamnk vaevlgyppm nyeyatfyt mynrkpvgky hiqvcttppc mlmsdsile alqkkligkv gettpdklt lieveclgac vnapnvqind nyedtakd ieeidelka gkipkpgprs grfscepagg lisleppkg pgfgvqagl

**MTS sequence**

Net charge of query sequence: +2

Analyzed region: 42

Number of basic residues in targeting sequence: 6

Number of acidic residues in targeting sequence: 0

Cleavage site: 44

Cleaved sequence:

MFFSAALARARAAGLTAHWGRHRNLHKTAMQNGAGGALFVHRD (SEQ ID NO: 42)

**3'UTR sequence (Accession # CC385433; SEQ ID NO: 43)**

```
cttgtsga actgaaata tgcactaga gaastaat mtgagttcc aatctaagta aaaaaaa aaaaaaa aaaaaaa aaaaaaa aaaaaaa aaaa
```
Mitochondrial aldehyde dehydrogenase 2 precursor (ALDH2)
Accession number: NP_000681

Protein sequence: 517 aa (SEQ ID NO: 54)
mrlaarrfgp rigrlisaa atqavpapnq qpevfcnqiif innnewhdavs rktfptvnpvs
tgevicqvae gkdedvdkaq kaaraafqalg spwrrmdash rgrlnrlad lidrtyla
altdnkgp yvigsylvdld mvikcrryaa gwadkyhgkt lpigdgffsy trhepvvgcg
qiipnwfll mqawklqpal atgqvnvmkvk aqotpnlaly vannikeaqf ppgvnnivpg
fgptagaalia shedvdkvaf tgssteigvgi qvaagssnik rytlelggks pnimsadmd
dwavezalfa lfnmgqccc agsttvqved iydefversv araksvvgn plfskelagpp
qvddegikki igyintgkqg gaklccqaggi aadrgyfipq tfgcdvqgdm ltkkeelgpp
vmqiikfkk eiivgranns tyglaaavft kdldkanyls qaiqagtvv ncyorvagaq
pfgykmsgs grelgeygld aytevkxv kvyqkns

MTS sequence
Net charge of query sequence: -1
Analyzed region: 32
Number of basic residues in targeting sequence: 5
Number of acidic residues in targeting sequence: 0
Cleavage site: 25
Cleaved sequence: MLRAARFGPRPLGRRLSAAATQA (SEQ ID NO: 44)

3'UTR sequence (Accession # CC369460; SEQ ID NO: 45)
gaactgtca aagtctcttc tgtccgccatt gatgqaaggt tcgcgcaagat cagcaacaa 60
accaagaaaa atgcatcttg cgtgctgat etgtgaaag gcagatgtttct cctcaaaat 120
cctcgagtc aagaagatct ttagaattgta atgtataaac atgytgggttt ggctgagggct 180
aagagatata gggacttctt ttttccgccc aatgctttgct tagcttttcttag gattttttt 240
aagaaaaatga ttcgaatgtgg tttcttcttt ccgtgaaagg tctctataact caggttttat 300
aggggaagaa aagagatatttt tgtttaaatat tctcccccatt aagcagactg cttccctcctg 360
cctttatatg ttggtctttaaa ttctttataa aaaggctctgt cttttaaaat aaaaaa 420
aaaaaaàaaaaa aaaaaaaàaaaaa aaaaaaaàaaaaa 450

FIGURE 11 G
Heme A:farnesyltransferase (COX10)
Accession number: NP_001294

Protein sequence: 443 aa (SEQ ID NO: 55)
maasplhls rltgcvggs wyvlertig dsphkhflh mvmnkqwif qhfsflkrmy
vqtnhrshq qvpkppeap splekstsq qakaeyerm plspsls krpinkele
lpepsevieds ivdyketkee krwkmldqv ydlpgilar skikitalv slaagfala
pppiddwpclf ltsvgtglas caansinqfg eypdfsnmmnr tknprlvqgg ispllavsf
ccavpgvai litgynplgl alfhnflyn tccyptklti siarntwvg vgaipvpgmgw
.taagsldag aflggiyq wtqphfnals wgredyrs gypcmmsvthp glcrvairh
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llillilmit rnpoggdapp pps

MTS sequence
Net charge of query sequence: +15
Analyzed region: 24
Number of basic residues in targeting sequence: 1
Number of acidic residues in targeting sequence: 0
Cleavage site: 21
Cleaved sequence: MAASPHTLSRLLLTGCVGGS (SEQ ID NO: 46)

3'UTR sequence (Accession # CC352261; SEQ ID NO: 47)
gttgttattgt atcccctccc aasacttgct gtaaatatgc egtttttttt ccctcaagagc t ... 60
cttcctctac atattttgct tccattttct tgttatttac cta ... 120
gaaacgagaa aggcaagggc ccttcgcctc tgcctgcgc a ... 180
aggtggcctgg atccttcggt cgcggcgctg ggcaatggtc ... 240
tgtctctccaa cccctctctcc caataattct aacagcgcgc ... 300
taclctcagc gttgtacgga gacccacggct cggagccagt ... 360
gggacgcac acacgaacct tggcagcggc agcaagctgc ... 420
aaaaaataa aaggaagggg gacaggaatg gggagagatc ctggtctacq ... 480
tagaacctct ttcgtctggtg ttcctctgct ccctcaagagc aa ... 540
caggaatcatt tataattgct ggacgacgct cgcctgatcg ... 600
ttggtgctagt tagtggctgt tggccgtggc ttc ... 660
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tcgagagtt ctgacgactg tggccctaga ctcctctctc ... 900
tgcacgcct ctgttctgcct tccctctctc aagaaagctg ... 960
tgcacgcct ctgttctgcct tccctctctc aagaaagctg ... 1020
aaagcaaggg gccgctgtat ctttcctctc gttgtgctgt ... 1080
ttggtgctagt tagtggctgt tggccgtggc ttc ... 1140
acacgtctct ttttctctct aacagtctgt ttttctctct ... 1200
tgcacgcct ctgttctgcct tccctctctc aagaaagctg ... 1260
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cttcctctac atattttgct tccattttct tgttatttac cta ... 120

FIGURE 11 H
Adenylate Kinase 2 (AK2)
Accession number: P54819

Protein sequence: 239 aa (SEQ ID NO: 56)
mapspypaap epyp gira lpqgpagagkt qapralrnf vchlatg-mdi ramvagsr
kkkikatmka gklvdemrr elieknilp lckngflig fprvqrqm lldkevke
kidsvi fpsi pdsliir fgrlphkgsr syheefnppk epmkddltge plirsdnee
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MTS sequence
Net charge of query sequence: +1
Analyzed region: 8
Number of basic residues in targeting sequence: 0
Number of acidic residues in targeting sequence: 0
Cleavage site:
Cleaved sequence:

3'UTR sequence (Accession # CC221872; SEQ ID NO: 57)
tatcagagac cccgcagcga ctgcaaacgt gctctcatcc cccgcgcgcag atctctgctc 60
atatcttcgq qccagagcgg gaaagtcttc tggcgagga gggcttgtga 120
aggggtccag qaaatccttg gaaatcagcc atgtaccttt tagctgagca gttttttttt 180
acatagggta agagttttaa aattgttaag gagaaaaatt aaattttttaa aagccatgg 240
aggggtcagc tgtccagcag cagcgcagc aagttttttt ttttttttttttttttttttttt 300
catttataag tgtccagcag cagcgcagc aagttttttt ttttttttttttttttttttttt 360
gaaacccaatg ggtctccagac caagatcaca gaaatcctaa aatttttttt tttttttttttt 420
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aataagaaaa aataaaaaaa aa

FIGURE 11 I
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**Controle**

**NDI muté**

**FIGURE 18**
IMPORTATION OF MITOCHONDRIAL PROTEIN BY AN ENHANCED ALLOTOPIC APPROACH

FIELD OF THE INVENTION

[0001] The present invention relates to the field of cell biology, molecular genetics, and medicine. It more particularly relates to the importation of proteins into the mitochondrion of animal and human cells.

BACKGROUND OF THE INVENTION

[0002] Mitochondria occupy a central position in the overall metabolism of eukaryotic cells; hence the oxidative phosphorylation (OXPHOS), the Krebs’s cycle, the urea cycle, the heme biosynthesis and the fatty acid oxidation take place within the organelle. Recently, another major role for mitochondria in determining the cellular life span was established, as they are recognized to be a major early mediator in the apoptotic cascade. Mitochondria are also a major producer of reactive oxygen species (ROS) causing oxidative stress and therefore inducers of cell death.

[0003] Primary defects in mitochondrial function are implicated in over 120 diseases and the list continues to grow, they encompass an extraordinary assemblage of clinical problems, commonly involving tissues that have high energy requirements, such as retina, heart, muscle, kidney, pancreas and liver. Their incidence is estimated of 1 in 5,000 live births. Indeed, combining epidemiological data on childhood and adult mitochondrial diseases suggests this prevalence as minimum, and could be much higher. Therefore, mitochondrial pathologies are considered among the most common genetically determined diseases, and are a major health issue since they remain inaccessible to both curative and palliative therapies.

[0004] Mitochondrion is assembled with proteins encoded by genes distributed between mitochondrial and nuclear genomes. These genes include those encoding the structural proteins of the respiratory chain complexes I-V, their associated substrates and products, the proteins necessary for mitochondrial biogenesis, the apparatus to import cytoplasmically synthesized precursors and the proteins necessary for mitochondrial assembly and turnover. Studies leading to the identification of genes involved in mitochondrial disorders have made considerable progress in the last decade. Indeed, numerous mutations in both mitochondrial DNA and a number of nuclear genes have been reported in association with a striking diversity of clinical presentations.

[0005] Approximately half of human mitochondrial disorders are caused by pathogenic point mutations of mtDNA, one-third of which are located in coding genes. There is currently no treatment for any of these disorders, a possible therapeutic approach is to introduce in the nucleus a wild-type copy of the gene mutated in the mitochondrial genome and import normal copies of the gene product into mitochondria from the cytosol. This approach has been termed “alloptic expression”.

[0006] There have already some reports describing that engineered nucleus-localized version of some mtDNA genes could be expressed in mammalian cells. For example, in a Leigh’s disease case, a plasmid was constructed in which the mitochondrial targeting signal of the nuclear encoded COX8 gene was appended to a re-coded mitochondrial ATP6 gene, mutated in patients. Stably transfected cells from patients present an improvement of growth in galactose medium and a mild increase in ATP synthesis, however the amount of Atp6 protein imported into mitochondria was relatively low (18.5%), implying that the precursor was not imported efficiently (Manfredi, G., et al., Rescue of a deficiency in ATP synthesis by transfer of MTATP6, a mitochondrial DNA-encoded gene to the nucleus. Nature Genet., 2002, 30: p. 394-399).

[0007] Oco-Cassio and co-workers have demonstrated that allotropic expression of apocytochrome b and ND4 into Cos-7 and HeLa cells, did not lead to an efficient mitochondrial import of these proteins (Oco-Cassio, J., et al., Limitations of allotropic expression of mitochondrial genes in mammalian cells. Genetics, 2003, 165: p. 707-720).

[0008] Hence, today important limitations are found to the allotropic expression as a therapeutic approach and require optimization to overcome the significant hurdles before it can be applied in genetic therapy.

[0009] One hypothesis that can explain the poor import ability of the mitochondrial protein is its high hydrophobicity. Thus, the precursor synthesized in the cytoplasm remains stuck on the outer mitochondrial membrane.

[0010] Mitochondria assembly depends on balanced synthesis of 13 proteins encoded by mtDNA with more than a thousand others encoded by nuclear DNA. As the vast majority of mitochondrial polypeptides are synthesized in the cytoplasm, there is the requirement for an efficient and specific protein targeting system. This process involves the transport of mRNAs from the nucleus to the surface of mitochondria.

[0011] The inventors examined the possibility that allotropic expression of DNA such as mtDNA could be optimized by a targeted localization of the mRNA to the mitochondrial surface.

SUMMARY OF THE INVENTION

[0012] Mitochondrial proteins are encoded by nucleic acids which are located in the mitochondrion, i.e. mitochondrial nucleic acids (mtDNA, mRNA), as well as by nucleic acids which originate from the nucleus, i.e. nuclear nucleic acids (nDNA, nRNA).

[0013] The inventors describe an enhanced allotypic approach for importation of proteins into the mitochondrion. The present invention provides means, including compositions and methods, which enable mitochondrial importation at enhanced efficiency and stability compared to prior art techniques. The means of the invention enable a targeted localization of the mRNA to the mitochondrial surface.

[0014] Compared to prior art techniques, the means of the invention enable the efficient and stable importation of protein into the mitochondrion of an animal or human in need thereof, such as an animal or human having a cellular dysfunction caused by one or several mutations in a gene encoding a mitochondrial protein.

[0015] The inventors demonstrate that mRNA sorting to the mitochondrial surface is an efficient way to proceed to such an allotypic expression, and that this mRNA sorting can be controlled by selecting appropriate mitochondrial-targeting sequence (MTS) and appropriate 3′UTR sequences. The CDS sequence which codes for the protein to be delivered into the mitochondrion is guided by these appropriate MTS and 3′UTR sequences from the nuclear compartment to the mitochondrial-bound polysomes (where the CDS is translated), and aids in an efficient translocation of a mature functional protein into the mitochondria.
The inventors demonstrate that, to obtain a stable therapeutically-effective importation, both an appropriate MTS and an appropriate 3'UTR should preferably be used.

Appropriate MTSs and 3'UTR sequences correspond to those of nuclearily-transcribed mitochondrially-targeted mRNAs. If a vector is used, it is preferred that it does not contain any 3'UTR which would correspond to the 3'UTR of a nuclearily-transcribed but not-mitochondrially-targeted mRNAs. To the best of the inventors’ knowledge, all commercially-available vectors contain such a not-mitochondrially-targeted mRNA; it is then preferred to delete this inappropriate 3'UTR from the vector before use as mitochondrial importer.

The means of the invention are especially adapted to animal and human cells, and more particularly to mammalian cells. They give access to therapeutically-effective means for such cells.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A and 1B: Map and Sequence of COX10 MTS-nATP6, SOD2MTS-nATP6, COX10 MTS-nATP6-COX10 3'UTR, SOD2MTS-nATP6-SOD2 3'UTR Obtained in the pCMV-Tag 4A Vector.

FIG. 1A. The four constructs are schematically represented.

FIG. 1B. The COX10 MTS-nATP6 and SOD2MTS-nATP6 are introduced at the EcoRI restriction site of the pCMV-tag4A vector. EcoRI restriction sites are framed. The ATG of SOD2 MTS, COX10 MTS and nATP6, are bold and underlined. The COX10 3'UTR and the SOD2 3'UTR are inserted at the PvuI and MluI restriction sites, represented in bold. FLAG tag epitope is in italics.

FIG. 2: RT-PCR Analyses of RNAs Purified from HeLa Transfected Cells

100 ng of total RNAs were reverse-transcribed and subjected to 30 cycles of PCR amplification, 10% of amplified product were subjected to agarose electrophoresis.

1: Transiently transfected cells with the COX10 MTS-nATP6 vector (SV40 3'UTR).
2: Transiently transfected cells with COX10 MTS-nATP6-COX10 3'UTR vector.
3 and 4: Transfection experiment but in this case it represents the stably transfected cells.
5: HeLa transfected cells with the empty pCMV-tag4A vector.
6: HeLa cells.

Specific oligonucleotide primers were used to detect hybrid ATP6 mRNA in transfected cells, for the MTS COX10-ATP6 product MTs COX10 and ATP6 ORF 3' were used (cf. Table 2 below, in example 1). For the amplification of the COX10-ATP6 3'UTR the ATP6 ORF 5' primer and the 3' UTR COX10 3' Primer were used (cf. Table 2 below, in example 1). As internal control, the steady-state levels of COX6 mRNA were examined in all the RNA preparations using both COX6 primers shown in said Table 2.

FIG. 3: Subcellular Localization of the Recoded ATP6 Protein in HeLa Cells

Stably transfected cells with either COX10 MTS-nATP6 (SV40 3'UTR) or COX10 MTS-nATP6-COX10 3'UTR vector were visualized by indirect immunofluorescence using antibodies to Flag and ATP synthase subunit beta. The punctuate pattern of Flag antibody staining indicates that the fusion ATP6 protein is efficiently transported to mitochondria in vivo, since the same pattern of mitochondria labeling was observed with the beta subunit of ATP synthase.

FIGS. 4A and 4B: nATP6 Proteins are Efficiently Imported into Mitochondria In Vivo.

FIG. 4A: Proteins were extracted from HeLa cells and HeLa transfected cells (COX10 MTS-nATP6-SV40 3'UTR or COX10 MTS-nATP6-COX10 3'UTR vectors) and assayed for import in the absence and presence of proteinase K (PK). Proteins were treated with 200 μg/ml of proteinase K at 0° C for 30 min. Then, they were separated on 4-12% polyacrylamide SDS gel and transferred into a nitrocellulose membrane. The resulting blot was probed with mouse monoclonal anti-ATP synthase subunit alpha or mouse monoclonal anti-Flag M2 antibodies.

FIG. 4B: Histograms of the amount of COX10 MTS-nATP6-Flag and Atp synthase subunit alpha with or without proteinase K. Signals from immunoblots were scanned and quantified by the MultiAnalyzer System (Bio-Rad). The amount of the mature ATP6 protein insensitive to proteinase K proteolysis is approximately 185% higher in cells transfected with COX10 MTS-nATP6-COX10 3'UTR vector compared to cells expressing the COX10 MTS-nATP6 without COX10 3'UTR but with the SV40 Poly A signal. Besides, the amount of the mature form of the recoded ATP6 protein inside mitochondria is very similar to the one measured for the naturally imported ATP synthase subunit alpha, confirming that recoded ATP6 proteins are efficiently translocated into the organelle.

FIGS. 5A and 5B: Map and Sequence of COX10 MTS-nND1, COX10 MTS-nND4, COX10 MTS-nND1-COX10 3'UTR, COX10 MTS-nND4-COX10 3'UTR Obtained in the pCMV-Tag 4A Vector.

FIG. 5A. The four constructs are schematically represented.

FIG. 5B. The COX10 MTS-nND1 and COX10MTS-nND4 are introduced at the XhoI/Sall restriction sites of the pCMV-tag4A vector. XhoI and Sall restriction sites are framed. The ATG of COX10 MTS, nND1 and nND4, are bold and underlined. The COX10 3'UTR is inserted at the PvuI and MluI restriction sites, represented in bold. FLAG tag is in italics.

FIG. 6: Immunocytochemistry of G3460A LHON Fibroblasts

The fusion protein was visualized by indirect immunofluorescence using antibodies to Flag. Indicative of mitochondrial import, cells transfected with both COX10 MTS-nND1-SV40 3'UTR or COX10 MTS-nND1-COX10 3'UTR vectors exhibited a typically punctuate staining pattern, also observed with the beta subunit of ATP synthase, which localize in vivo to the inner mitochondrial membrane. In contrast, cells transfected with the empty pCMV-Tag 4A vector exhibited a very low intensity and diffuse cytoplasmic staining when antibodies to Flag were used.

FIG. 7: Immunocytochemistry of G11778A LHON Fibroblasts

The fusion protein was visualized by indirect immunofluorescence using antibodies to Flag. Cells transfected with either COX10 MTS-nND4-SV40 3'UTR or COX10 MTS-nND4-COX10 3'UTR vectors exhibited a typically punctuate staining pattern, also observed with the beta subunit of ATP synthase, which localize in vivo to the inner mitochondrial membrane. This data indicates that ND4 is efficiently imported into mitochondria. In contrast, cells trans-
affected with the empty pCMV-Tag 4A vector exhibited a very low intensity and diffuse cytoplasmic staining when antibodies to Flag were used.

**[0042]** FIG. 8: Growth in Glucose-Free Medium of Non-Transfected Fibroblasts with the G3460A Mutation and Transfected Fibroblasts with the MTS COX10-nND1-COX10 3'UTR Vector

**[0043]** Fibroblasts from LHON patients presenting the G3460A mutation were stably transfected with the MTS COX10-nND1-COX10 3'UTR vector and examined for their ability to growth on DMEM medium supplemented with 10 mM galactose. Non-transfected fibroblasts (LHON G3460A ND1) show a severe growth defect on galactose medium, the ability to grow on galactose was significantly improved when the recoded nND1 protein is expressed in stably transfected cells (LHON G3460A ND1+MTSCOX10-nND1). Cells were photographed after 6 day culture.

**[0044]** FIG. 9: Recoded CDS of mtDNA (SEQ ID NO: 27-29)

**[0045]** FIG. 9 shows the human nuclear acid coding sequence of the mitochondrial ATP6, ND1, ND4, recoded according to the universal genetic code (nATP6, nND1, nND4 of SEQ ID NO:27, 28 and 29, respectively. The recoded ND1 and ND4 which are shown in FIG. 9 also take into account the preferred human codon usage (see example 2 below).

**[0046]** FIG. 10: Illustrative Human Co-Translational MTS and 3'UTR (SEQ ID NOS: 30, 47, 31, 60)

**[0047]** FIG. 10 shows the sequence of human COX10 MTS (SEQ ID NO: 30), human COX10 3'UTR (SEQ ID NO: 47), human SOD2 MTS (SEQ ID NO: 47), and human SOD2 3'UTR (SEQ ID NO: 60).

**TABLE 5**

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<th>Nucleic acid MTS</th>
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<tr>
<td>SOD2</td>
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**[0048]** FIGS. 11A-111 show the protein sequence coded by illustrative human mitochondrially-targeted mRNA, as well as their respective MTS peptide sequences and their respective 3'UTR sequences. ATCC accession number is indicated for each of these protein sequences.

**[0049]** ACO2~Aconitase;  
**[0050]** SOD2~Mitochondrial Superoxide dismutase;  
**[0051]** ATP5~B~p synthase subunit beta;  
**[0052]** UQCRFS1~Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1;  
**[0053]** NDUFV1~NADH-ubiquinone oxidoreductase 51 kDa subunit, mitochondrial precursor (Complex I-51KDF (Cl-51KDF) (NADH dehydrogenase flavoprotein 1);  
**[0054]** NDUFV2~NADH dehydrogenase (ubiquinone) flavoprotein 2, 24 kDa;  
**[0055]** ALDH2~Mitochondrial aldehyde dehydrogenase 2 precursor;  
**[0056]** COX10~Heme A:farnesyltransferase;  
**[0057]** AK2~Adenylyl Kinase 2.

**[0058]** SEQ ID NO are as follows:

**TABLE 6**

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**[0059]** FIGS. 12A, 12B, 12C: Subcellular Distribution of Hybrid ATP6 mRNAs in HeLa Cells

**[0060]** A. Total RNAs extracted from cells expressing the SOD2<sup>MTS</sup>ATP6-3'UTR<sup>S440</sup> (S.T 1 and S.T 2) or the SOD2<sup>MTS</sup>ATP6-3'UTR<sup>S202</sup> (S.T 3 and S.T 4) vectors were subjected to RT-PCR analysis to reveal amounts of hybrid ATP6 (SOD2<sup>MTS</sup>ATP6) mRNAs and endogenous SOD2, ATP6 and COX6c mRNAs. The amount of RNAs used for the reverse transcription, PCR conditions and specific oligonucleotides used for each gene are summarized in Table 9.

**[0061]** B. RNAs were purified from mitochondrial-bound polysomes (M-P) and free-cytoplasmic polysomes (F-P) of stably transfected cell lines with either SOD2<sup>MTS</sup>ATP6-3'UTR<sup>S440</sup> (S.T 1 and S.T 2) or SOD2<sup>MTS</sup>ATP6-3'UTR<sup>S202</sup> (S.T 3 and S.T 4) vectors and subjected to RT-PCR analysis. The abundance of endogenous ATP6, SOD2 and COX6c mRNAs was determined in each polysomal population using the conditions shown in Table 9.

**[0062]** C. Densitometric analyses were performed using the Quantity One Biorad software system.

**[0063]** The difference between the amounts of hybrid ATP6 mRNAs in cells expressing respectively SOD2<sup>MTS</sup>ATP6-3'UTR<sup>S440</sup> or SOD2<sup>MTS</sup>ATP6-3'UTR<sup>S202</sup> constructions was significant according to the paired Student’s t-test (F<0.0054, n=6).

**[0064]** FIG. 13: Subcellular Localization of the Recoded ATP6 Protein In Vivo

**[0065]** Stably transfected cells with either the empty pCMV-Tag4A vector: SOD2<sup>MTS</sup>ATP6-3'UTR<sup>F440</sup> or SOD2<sup>MTS</sup>ATP6-3'UTR<sup>S202</sup> plasmids were visualized by indirect immunofluorescence using antibodies to Flag and ATP synthase subunit α. For each cell type visualized, a merged image in association with DAPI staining is shown at the right panel. Indicative of the mitochondrial localization of recoded ATP6 proteins, cells transfected with either SOD2<sup>MTS</sup>ATP6-3'UTR<sup>S440</sup> or SOD2<sup>MTS</sup>ATP6-3'UTR<sup>S202</sup> plasmids showed a significant colocalization of both Flag and ATP synthase α signals. In contrast, cells transfected with the empty vector exhibited a low diffuse cytoplasmatic staining.

**[0066]** FIGS. 14A, 14B, 14C: Recoded ATP6 Proteins are Efficiently Imported into Mitochondria In Vivo.

**[0067]** A. Six independent mitochondria purifications were performed with cells stably transfected with either SOD2<sup>MTS</sup>ATP6-3'UTR<sup>S440</sup> or SOD2<sup>MTS</sup>ATP6-3'UTR<sup>S202</sup> plasmids and subjected to Western blot analysis. Signals for the ATP6 precursors and mature forms were scanned and quantified by the Quantity One System (Bio-Rad). No significant differences between the amounts of the precursor and the mature form of the recoded ATP6 proteins were observed in each cell line examined.
B. Upper panel: Schematic representation of mitochondrial import intermediates. The hydrophobic passenger protein can be trapped en route to the matrix. In this step, the protein can be blocked or represented an intermediate of translocation. This doesn’t prevent the cleavage of the MTS by a mitochondrial processing peptidase, the rest of the protein remains accessible to PK digestion and therefore if digested it becomes undetectable in the Western blot assay. The fraction of the protein completely translocated turns into a mature protein insensitive to PK located in the inner mitochondrial membrane. MM: mitochondrial matrix; OM: outer membrane; MIM: mitochondrial intermembrane space, TOM: Translocase of the Outer Membrane, TIM: Translocase of the Inner membrane.

Middle panel: Mitochondria extracted from transfected cells with either the empty pCMV-Tag 4A vector, SOD2MTS-ATP6-3’UTR or SOD2ATP6-3’UTR plasmids were subjected to Western blot assays. 20 mg of proteins were treated with 150 µg/ml of PK at 0°C for 30 minutes and subjected to immunoblotting analysis using anti-ATP synthase subunit α and anti-Flag M2 antibodies. Densitometric analyses of experiments performed with six independent mitochondrial purifications were represented at the lower panel. We normalized values measured for the signal of the mature form of ATP6 resistant to PK with ATPα signal revealed after PK digestion. We then compared the value obtained for cells expressing either the SOD2MTS-ATP6-3’UTR or the SOD2-ATP6-3’UTR plasmids. Signals from Western blots were scanned and quantified by the Quantity One System (Bio-Rad). The difference between the amounts of fully mitochondrial translocated ATP6 protein in cells expressing respectively SOD2MTS-ATP6-3’UTR or SOD2MTS-ATP6-3’UTR constructions was significant according to the paired Student’s t-test (P<0.0022, n=6).

C. 20 µg of mitochondria isolated from cells stably transfected with SOD2MTS-ATP6-3’UTR vector treated with 150 µg/ml of PK and 1% Triton X100 at 0°C for 30 min and subsequently subjected to Western analysis.

FIG. 15: Mitochondrial Import Ability of ATP6 Proteins Based on the Mesohydrophobicity Index.

A plot developed by Charos and Vincens was used to measure mitochondrial import ability of fusion ATP6 proteins. By this approach, the fusion SOD2MTS-ATP6 protein would not be importable. Mesohydrophobicity, which is the average regional hydrophobicity over a 69 amino acid region, was calculated using MitoProtI. Values obtained are the following: ATP6: 1.41; SOD2MTS-ATP6: 1.41; COX8MTSATP6: 1.41; SOD2: -1.26; COX8: -1.63.

FIG. 16: rescue of NARP cells; survival rate on galactose medium of NARP cells (mutated ATP6), and of NARP cells transfected by a SOD2 MTS-ATP6 vector (SOD2 MTS-ATP6-SV40 3’UTR), or by a vector of the invention (SOD2 MTS-ATP6-SOD2 3’UTR); see also example 3, table 11.

FIG. 17: rescue of LHON fibroblasts; survival rate on galactose medium of LHON fibroblasts (mutated ND1), and of LHON fibroblasts transfected by a COX10 MTS-ND1-SV40 3’UTR vector, or by a vector of the invention (COX10 MTS-ND1-COX10 3’UTR); see also example 4, table 12.

FIG. 18: mitochondrial distribution in retinal ganglion cells (RGC) transfected with the mutated version of ND1.

FIG. 19: rescue of NARP cells; anti-Flag, Mitotracker and merged+DAPI staining of NARP cells transfected either with SOD2 MTS-ATP6-SV40 3’UTR, or with SOD2 MTS-ATP6-SOD2 3’UTR.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use and control of mRNA sorting at the surface of mitochondria.

Schematically, the present invention relates to the use of nucleic acid sequences corresponding to a co-translational MTS and of a co-translational 3’UTR, for guiding a desired mRNA (which codes a desired mitochondrial protein) from the nucleus to the mitochondria-bound polysomes, and for inducing the effective translocation of the translated protein into the mitochondrion.

By “co-translational”, it is herein referred to a nuclearily-encoded mitochondrially-targeted pathway.

Mitochondrion-Targeting Sequences (MTS):

Sequences known as mitochondrion-targeting signal or mitochondrial targeting signal are referred to as MTS by the skilled person.

A MTS sequence can be identified within a protein or nucleic acid sequence by a person of ordinary skill in the art.

Most mitochondrion-targeting peptides consist of a N-terminal pre-sequence of about 15 to 100 residues, preferably of about 20 to 80 residues. They are enriched in arginine, leucine, serine and alanine. Mitochondrial pre-sequence show a statistical bias of positively charged amino acid residues, provided mostly through arginine residues; very few sequences contain negatively charged amino acids. Mitochondrion-targeting peptides also share an ability to form an amphiphilic alpha-helix.


Software is available to the skilled person to identify the MTS of a given sequence. Illustrative software notably comprises the MitoProt® software, which is available e.g. on the web site of the Institut für Humangenetik; Technische Universität München, Germany. The MitoProt® software calculates the N-terminal protein region that can support a Mitochondrial Targeting Sequence and the cleavage site. The identification of the N-terminal mitochondrial targeting peptide that is present within a protein gives a direct access to the nucleic acid sequence, i.e. to the MTS (e.g. by reading the corresponding positions in the nucleic acid coding for said protein).

SEQ ID NOs are as follows:

<table>
<thead>
<tr>
<th>Illustrative human mRNA which are nuclearly-encoded but mitochondrially-targeted</th>
<th>MTS peptide</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACO2</td>
<td>SEQ ID NO: 32</td>
<td>11A</td>
</tr>
<tr>
<td>SOD2</td>
<td>SEQ ID NO: 34</td>
<td>11B</td>
</tr>
<tr>
<td>ATP5b</td>
<td>SEQ ID NO: 36</td>
<td>11C</td>
</tr>
<tr>
<td>UQCRFS1</td>
<td>SEQ ID NO: 48</td>
<td>11D</td>
</tr>
<tr>
<td>ND5U1</td>
<td>SEQ ID NO: 40</td>
<td>11E</td>
</tr>
<tr>
<td>ND5U2</td>
<td>SEQ ID NO: 42</td>
<td>11F</td>
</tr>
<tr>
<td>ALDH2</td>
<td>SEQ ID NO: 44</td>
<td>11G</td>
</tr>
<tr>
<td>COX10</td>
<td>SEQ ID NO: 46</td>
<td>11H</td>
</tr>
</tbody>
</table>

3'UTR:

The 3'UTR of a RNA molecule is defined as the fragment of this RNA molecule that extends from the STOP codon to the end of the molecule. According to the universal genetic code, there are three possible STOP codons: TGA, TAA, TAG.

An online database gives direct access to 3'UTR sequences.

Illustrative 3'UTR sequences which can be used in accordance with the invention are shown in FIGS. 11A, 11B, 11C, 11D, 11E, 11F, 11G, 11H and 11I (Accession numbers of these sequences are also indicated).

SEQ ID NOs are as follows:

<table>
<thead>
<tr>
<th>Illustrative human mRNA which are nuclearly-encoded but mitochondrially-targeted</th>
<th>3'UTR</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACO2</td>
<td>SEQ ID NO: 33</td>
<td>11A</td>
</tr>
<tr>
<td>SOD2</td>
<td>SEQ ID NO: 35</td>
<td>11B</td>
</tr>
<tr>
<td>ATP5b</td>
<td>SEQ ID NO: 37</td>
<td>11C</td>
</tr>
<tr>
<td>UQCRFS1</td>
<td>SEQ ID NO: 39</td>
<td>11D</td>
</tr>
<tr>
<td>ND5U1</td>
<td>SEQ ID NO: 41</td>
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<tr>
<td>ND5U2</td>
<td>SEQ ID NO: 43</td>
<td>11F</td>
</tr>
<tr>
<td>ALDH2</td>
<td>SEQ ID NO: 45</td>
<td>11G</td>
</tr>
<tr>
<td>COX10</td>
<td>SEQ ID NO: 47</td>
<td>11H</td>
</tr>
<tr>
<td>AK2</td>
<td>SEQ ID NO: 57</td>
<td>11I</td>
</tr>
</tbody>
</table>

Vectors of the Invention:

The present invention relates to a vector which is adapted to the efficient and stable delivery of a protein into the mitochondrion of an animal or human cell, preferably a mammalian cell, most preferably a human cell.

The vector of the invention can be produced in the form of a recombinant vector. Advantageously, the vector of the invention is an expression vector.

A vector of the invention comprises:

- at least one nucleic acid sequence encoding a mitochondrion-targeting signal (also referred to as: MTS nucleic acid sequence),
- at least one nucleic acid sequence which encodes said protein to be delivered, in accordance with the universal genetic code (also referred to as: CDS), and at least one 3' nucleic acid sequence.
- Said at least one MTS nucleic acid sequence is a co-translational MTS nucleic acid sequence, or a conservative fragment or variant thereof.
mRNA to the surface of a mitochondrion in a cell collected from a healthy animal or human being, or in a normal animal or human cell, or

[0117] the cDNA sequence of such a MTS RNA sequence, or
[0118] a DNA sequence coding for such a MTS RNA sequence in accordance with the universal genetic code, or
[0119] a conservative variant or fragment of such a RNA or cDNA or DNA MTS sequence, which derives therefrom by deletion and/or substitution and/or addition of one or several nucleotides, but has retained a mitochondrion-targeting function.

[0120] Preferably, said at least one MTS nucleic acid sequence is:

[0121] the cDNA sequence of a MTS of a nuclearly-encoded mitochondrionally-targeted mRNA, or
[0122] a conservative variant or fragment of such a cDNA sequence, which derives therefrom by deletion and/or substitution and/or addition of one or several nucleotides, but has retained a mitochondrion-targeting function.

[0123] Said at least one 3' nucleic acid sequence is:

[0124] the 3'UTR sequence of a nuclearly-encoded mitochondrionally-targeted mRNA, preferably the 3'UTR sequence of a naturally-occurring nuclearly-encoded mitochondrionally-targeted mRNA, or
[0125] the cDNA sequence of such a 3'UTR sequence, or
[0126] a DNA sequence coding for such a 3'UTR sequence in accordance with the universal genetic code, or
[0127] a conservative variant or fragment of such a RNA or cDNA or DNA 3'UTR sequence, which derives therefrom by deletion and/or substitution and/or addition of one or several nucleotides, and which, when replacing the wild-type 3'UTR of said nuclearly-encoded mitochondrionally-targeted mRNA, still allows for a mitochondrial targeting of the resulting mRNA.

[0128] In other words, said at least one 3' nucleic acid sequence is:

[0129] the RNA sequence of the 3'UTR of a nuclearly-transcribed mitochondrionally-targeted mRNA, i.e. the RNA sequence of the 3'UTR of a (preferably naturally-occurring) nuclearly-transcribed RNA which is targeted to the surface of a mitochondrion in a cell collected from a healthy animal or human being, or in a normal animal or human cell, or
[0130] the cDNA sequence of such a 3'UTR sequence, or
[0131] a DNA sequence coding for such a 3'UTR sequence in accordance with the universal genetic code, or
[0132] a conservative variant or fragment of such a RNA or cDNA or DNA 3'UTR sequence, which derives therefrom by deletion and/or substitution and/or addition of one or several nucleotides, and which, when replacing the wild-type 3'UTR of said nuclearly-encoded mitochondrionally-targeted mRNA, still allows for a mitochondrial targeting of the resulting mRNA.

[0133] Preferably, said at least one 3' nucleic acid sequence is:

[0134] the cDNA sequence of the 3'UTR sequence of a nuclearly-encoded mitochondrionally-targeted mRNA, or
[0135] a conservative variant or fragment of such a cDNA sequence, which derives therefrom by deletion and/or substitution and/or addition of one or several nucleotides, and which, when replacing the wild-type 3'UTR of said nuclearly-encoded mitochondrionally-targeted mRNA, still allows for a mitochondrial targeting of the resulting mRNA.

[0136] The resulting vector does not use a post-translation importation pathway, but uses a co-translation importation pathway from nucleus to said mitochondrion.

[0137] Preferably, said vector (inserted nucleic acid construct included) does not comprise any sequence which would be identical to:

[0138] the 3'UTR of a naturally-occurring mRNA which is a (preferably naturally-occurring) nuclearly-transcribed but non-mitochondrially-targeted mRNA, or
[0139] the cDNA sequence of such a 3'UTR sequence, or
[0140] a DNA sequence coding such a 3'UTR in accordance with the universal genetic code.

[0141] Preferably, said vector (inserted nucleic acid construct included) does not comprise any sequence which would be identical to:

[0142] the 3'UTR of a a mRNA which is not targeted to the surface of a mitochondrion, and preferably the 3'UTR of a naturally-occurring mRNA which is not targeted to the surface of a mitochondrion, or
[0143] the cDNA sequence of such a 3'UTR sequence, or
[0144] a DNA sequence coding such a naturally-occurring mRNA 3'UTR in accordance with the universal genetic code.

[0145] The present invention more particularly relates to a vector adapted to the efficient and stable delivery of a protein into the mitochondrion of a mammalian cell, which comprises:

[0146] at least one mitochondrion-targeting nucleic acid sequence (referred to as MTS nucleic acid sequence),
[0147] at least one nucleic acid sequence which encodes said protein in accordance with the universal genetic code (referred to as CDS sequence), and
[0148] at least one 3' nucleic acid sequence, which is located in 3' of said at least one MTS nucleic acid sequence and of said at least one CDS,
[0149] wherein said at least one MTS nucleic acid sequence is:

[0150] the cDNA sequence of a MTS of a nuclearly-encoded mitochondrionally-targeted mRNA, or
[0151] a conservative variant or fragment of such a cDNA sequence, which derives therefrom by deletion and/or substitution and/or addition of one or several nucleotides, but has retained a mitochondrion-targeting function.

[0152] wherein said at least one 3' nucleic acid sequence is:

[0153] the cDNA sequence of the 3'UTR sequence of a nuclearly-encoded mitochondrionally-targeted mRNA, or
[0154] a conservative variant or fragment of such a cDNA sequence, which derives therefrom by deletion and/or substitution and/or addition of one or several nucleotides, and which, when replacing the wild-type 3'UTR of said naturally-occurring mRNA, still allows for a mitochondrial targeting of the resulting mRNA,
[0155] wherein said vector does not comprise any sequence which would be identical to:

[0156] the 3′UTR of a naturally-occurring mRNA which is a nuclearly-transcribed but not-mitochondrially-targeted mRNA, or

[0157] the cDNA sequence of such a 3′UTR sequence, or

[0158] a DNA sequence coding for such a naturally-occurring mRNA 3′UTR in accordance with the universal genetic code,

[0159] whereby said vector does not use a post-translation importation pathway, but uses a co-translation importation pathway from nucleus to said mitochondrion.

[0160] Said at least one MTS nucleic acid sequence can e.g. be the MTS nucleic acid sequence of ACO2, or of SOD2, or of ATP5b, or of UQCRFS1, or of NDUFV1, or of NDUFV2, or of ALDH2, or of COX10.

[0161] Said at least one MTS nucleic acid sequence may thus code for a sequence of SEQ ID NO:32, or SEQ ID NO:34, or SEQ ID NO:36, or SEQ ID NO:38, or SEQ ID NO:40, or SEQ ID NO:42, or SEQ ID NO:44, or SEQ ID NO:46 (=the MTS peptide or polypeptide sequence of human ACO2, SOD2, ATP5b, UQCRFS1, NDUFV1, NDUFV2, ALDH2, COX10, respectively; see FIGS. 11A-11I).

[0162] Preferably, said at least one MTS nucleic acid sequence is the MTS nucleic acid sequence of ACO2, or of SOD2, or of ATP5b, or of COX10.

[0163] Said at least one MTS nucleic acid sequence may thus code for a sequence of SEQ ID NO:32, or SEQ ID NO:34, or SEQ ID NO:36, or SEQ ID NO:38, or SEQ ID NO:40, or SEQ ID NO:42, or SEQ ID NO:44, or SEQ ID NO:46 (=the MTS peptide or polypeptide sequence of human ACO2, SOD2, ATP5b, UQCRFS1, NDUFV1, NDUFV2, ALDH2, COX10, respectively).

[0164] Preferably, said at least one MTS nucleic acid sequence is SEQ ID NO:30, or SEQ ID NO:31 (MTS nucleic acid sequence of human COX10 and SOD2, respectively; see FIG. 10).

[0165] Said at least one 3′ nucleic acid sequence can be e.g. be:

[0166] the 3′UTR sequence of ACO2, or of SOD2, or of ATP5b, or of UQCRFS1, or of NDUFV1, or of NDUFV2, or of ALDH2, or of COX10, respectively.

[0167] the cDNA sequence of such a 3′UTR sequence, or

[0168] a DNA sequence coding for such a 3′UTR sequence in accordance with the universal genetic code.

[0169] Said at least one 3′ nucleic acid sequence may thus comprise or consist of those of SEQ ID NO:33, or SEQ ID NO:35, or SEQ ID NO:37, or SEQ ID NO:39, or SEQ ID NO:41, or SEQ ID NO:43, or SEQ ID NO:45, or SEQ ID NO:47, or SEQ ID NO:57 (=the sequences corresponding to the human 3′UTR of ACO2, SOD2, ATP5b, UQCRFS1, NDUFV1, NDUFV2, ALDH2, COX10, AK2, respectively; see FIGS. 10 and 11A-11I).

[0170] Preferably, said at least one 3′ nucleic acid sequence is:

[0171] the 3′UTR sequence of ACO2, or of SOD2, or of ATP5b, or of COX10, or of AK2, or

[0172] the cDNA sequence of such a 3′UTR, or

[0173] a DNA sequence coding for such a 3′UTR sequence.

[0174] Said at least one 3′ nucleic acid sequence may thus comprise or consist of SEQ ID NO:33, or SEQ ID NO:35, or SEQ ID NO:37, or SEQ ID NO:47, or SEQ ID NO:57 (=the sequences corresponding to the human 3′UTR of ACO2, SOD2, ATP5b, COX10, AK2, respectively; see FIGS. 10 and 11A-11I).

[0175] Preferably, said at least one 3′ nucleic acid sequence is SEQ ID NO:35 (human SOD2 3′UTR), or SEQ ID NO:47 (human COX10 3′UTR).

[0176] Said at least one CDS nucleic acid sequence can be a RNA, a cDNA or a DNA sequence. Preferably, said at least one CDS sequence is a cDNA sequence.

[0177] According to a very advantageous aspect of the invention, said at least one CDS may be any nucleic acid which codes for a protein that may be found useful for a mitochondrion. Contrary to prior art techniques, the technology of the invention is indeed not limited by the level of hydrophobicity of the encoded protein.

[0178] Said at least one CDS may thus be any nucleic acid coding for a mitochondrial protein. This nucleic acid may be a mitochondrial nucleic acid, or a nuclear nucleic acid coding for a mitochondrial protein.

[0179] Most preferably said at least one CDS sequence codes for a naturally-occurring functional mitochondrial protein, such as Cox1, Cox2, Cox3, Apt6, Apt8, Cytb, Nd1, Nd2, Nd3, Nd4, Nd41, Nd5, Nd6.

[0180] Preferably, said at least one CDS sequence is the sequence of a naturally-occurring mitochondrial nucleic acid, recoded in accordance with the universal genetic code.

[0181] The mitochondrial nucleic acids use a mitochondrial genetic code which is slightly different from the universal genetic code that is used by nuclear nucleic acids.

[0182] When the protein to be imported into said mitochondrion corresponds to a naturally-occurring mitochondrial protein, the naturally-occurring form of its nucleic acid sequence follows the mitochondrial genetic code.

[0183] When such a mitochondrial nucleic acid has to be inserted in the vector of the invention, the mitochondrial nucleic acid sequence has to be recoded in accordance with the universal genetic code, as the vector directs a co-translational importation process from nucleus to mitochondrion. Hence, a nuclear-encoded version of the mitochondrial nucleic acid sequence has to be created. This nuclear-encoded version can be produced by codon substitution in the mitochondrial nucleic acid, so as to replace those codons which are read by the mitochondrial genetic system with codons of the universal genetic code. For example, the mammalian UGA codon directs insertion of a tryptophan in mitochondria, but is a stop codon in the nuclear genetic code. Therefore, the UGA codon of a mitochondrial nucleic acid has to be replaced with UGG which codes for tryptophan in the universal genetic code.

<table>
<thead>
<tr>
<th>TABLE A</th>
</tr>
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<tr>
<td></td>
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<tr>
<td>UGA</td>
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<tr>
<td>AGG</td>
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<tr>
<td>AUA</td>
</tr>
</tbody>
</table>

[0184] Codon usage in mitochondria vs. the universal genetic code is described in Lewin, Genes V, Oxford University Press; New York 1994, the content of which being incorporated by reference.
 Codon substitutions notably include:

- UGA to UGG,
- AGA to UAA, UAG or UGA,
- AGG to UAA, UAG or UGA,
- AUA to AUG, CUG or GUG,
- AUU to AUG, CUG or GUG.

Said at least one CDS sequence may e.g. a nucleic acid sequence coding for Ap66, or Nd1, or Nd4, such as a nucleic acid sequence of ATP6, of or ND1, of or ND4, recoded in accordance with the universal genetic code (e.g. a sequence of SEQ ID NO:27, NO:28 or NO:29, see FIG. 9).

Said at least one CDS sequence may e.g. a nucleic acid sequence a nucleic acid sequence coding for Cox1, Cox2, Cox3, Ap8, Cyb, Nd2, Nd3, Nd41, Nd5, Nd6, such as a nucleic acid sequence of Cox1, Cox2, Cox3, ATP8, Cyb, ND2, ND3, ND41, ND5, ND6.

The description of the thirteen naturally-occurring mitochondrial nucleic acids can be found in Andrew et al. 1999 (Nat Genet. 1999 October; 23(2): 147).

Preferably, said recoding is made taking into account the preferred usage codon of said mammalian cell, and most preferably taking into account the human preferred usage codon.

When recoding mitochondrial nucleic acid according to the universal genetic code, it is according to the present invention very advantageous to take into account the preferred codon usage of the subject or patient, to which the vector or nucleic acid of the invention is to be administered.

Preferred codon usage principles, as well as examples of preferred codon usage for various organisms can e.g. be found in Klump and Maeder, 1991 (Pure & Appl. Chem., vol. 63, No. 10, pp. 1357-1366 “the thermodynamic basis of the genetic code”), the content of which is herein incorporated by reference. An illustrative preferred codon usage for human beings is shown in Table 3 below (see example 2).

Said at least one CDS sequence may e.g. the nucleic acid sequence of SEQ ID NO:28 or of SEQ ID NO:29 (i.e. a nucleic acid sequence of ND1 or of ND4, recoded in accordance with the universal genetic code, and taking into account the human preferred usage codon).

The vector of the invention may e.g. comprise:

- at least one SOD2 MTS nucleic acid sequence and at least one SOD2 3'UTR, or
- at least one COX10 MTS nucleic acid sequence and at least one COX10 3'UTR, or
- any combination of these MTS nucleic acid sequences and 3'UTR that the skilled person may find appropriate.

Such a vector may e.g. comprise a recoded ATP6, ND1 or ND4 as CDS.

The vector of the invention may e.g. comprise at least one sequence of SEQ ID NO:21 (COX10 MTS-re-coded ATP6-COX10 3'UTR), SEQ ID NO:22 (SOD2 MTS-re-coded ATP6-SOD2 3'UTR), SEQ ID NO:25 (COX10 MTS-re-coded ND1-COX10 3'UTR), SEQ ID NO:25 (COX10 MTS-re-coded ND4-COX10 3'UTR).

Alternatively, said at least one CDS sequence may be the nucleic acid sequence of a nuclear nucleic acid which encodes a functional mitochondrial protein, e.g., a naturally-occurring nuclear nucleic acid which encodes a functional mitochondrial protein.

More particularly, said at least one nuclear nucleic acid can be a nuclearly-transcribed mitochondrionally-targeted mRNA, or the cDNA sequence of such a mRNA, or the DNA sequence coding for such a mRNA.

More particularly, said at least one nuclear nucleic acid can be a nuclearly-transcribed mRNA which is not mitochondrionally-targeted, or the cDNA sequence of such a mRNA, or the DNA sequence coding for such a mRNA.

Said vector may further comprise one or several expression control sequences.

The selection of suitable expression control sequences, such as promoters is well known in the art, as is the selection of appropriate expression vectors (see e.g. Sambrko et al. “Molecular Cloning: A Laboratory Manual”, 2nd ed., vols. 1-3, Cold Spring Harbor Laboratory, 1989, the content of which is herein incorporated by reference).

Said vector may thus further comprise at least one promoter operably linked to said at least one MTS sequence, said at least one CDS sequence, said at least one 3' sequence.

Said promoter may e.g. be a constitutive promoter, such as e.g. a CMV promoter.

Said vector may further comprise a termination site.

Said vector may further comprise one of several of the following expression control sequences: insulators, silencers, IRES, enhancers, initiation sites, termination signals.

Said vector may further comprise an origin of replication.

Preferably, said promoter and said origin of replication are adapted to the transcription or infection of animal or human cells, preferably to the transcription or infection of human cells.

Said vector can e.g. a plasmid, or a virus, such as an integrating viral vector, e.g. a retrovirus, an adeno-associated virus (AAV), or a lentivirus, or is a non-integrating viral vector, such as an adenovirus, an alphasirus, a herpes simplex Virus (HSV).

Said vector may further comprise a nucleic acid coding for a detectable marker, such as a FLAG epitope or green fluorescent protein (GFP).

The present invention also relates to a process for the production of a vector of the invention, which comprises:

- providing a vector, and depleting from its original 3'UTR, if any,
- inserting in this vector at least one MTS nucleic acid sequence, at least one CDS sequence, and at least one 3' sequence as above-described.

As already-mentioned, said vector should preferably not comprise any sequence corresponding to the 3'UTR of a nuclearly-encoded but non-mitochondrially-targeted mRNA. To the best of the inventors’ knowledge, all commercially-available vectors contain such an inappropriate 3'UTR; according to the present invention, such a 3'UTR should hence be removed from the vector. It may e.g. be replaced by an appropriate 3' sequence corresponding to a nuclearly-encoded and mitochondrially-targeted mRNA.

Nucleic Acid Construct of the Invention:

The nucleic acid construct which is carried by the vector of the invention is also encompassed by the present invention. The present invention more particularly relates to a non-naturally occurring nucleic acid construct.

A non-naturally occurring nucleic acid construct of the invention comprises:

- at least one mitochondrion-targeting nucleic acid sequence (referred to as MTS nucleic acid sequence),
[0225] at least one nucleic acid sequence which encodes
said protein in accordance with the universal genetic
code (referred to as CDS sequence), and

[0226] at least one 3' nucleic acid sequence, which is
located in 3' of said at least one MTS nucleic acid
sequence and of said at least one CDS sequence.

[0227] Said at least one MTS nucleic acid sequence is:

[0228] the MTS RNA sequence of a naturally-encoded
mitochondrially-targeted mRNA, such as the MTS RNA
sequence of a naturally-occurring nearly-encoded
mitochondrially-targeted mRNA, or

[0229] the cDNA sequence of such a RNA, or

[0230] a DNA sequence coding for such a MTS RNA
sequence, or

[0231] a conservative variant or fragment of such a RNA
or DNA MTS sequence, which derives therefrom by
deletion and/or substitution and/or addition of one or
several nucleotides, but has retained a mitochondrion-
targeting function.

[0232] Said at least one 3' nucleic acid sequence is:

[0233] the 3'UTR sequence of a naturally-encodedmito-
chondrially-targeted mRNA, such as the 3'UTR
sequence of a naturally-occurring nearly-encoded
mitochondrially-targeted mRNA, or

[0234] the cDNA sequence of such a RNA, or

[0235] a DNA sequence coding for such a 3'UTR
sequence, or

[0236] a conservative variant or fragment of such a RNA
or DNA 3'UTR sequence, which derives therefrom by
deletion and/or substitution and/or addition of one or
several nucleotides, and which, when replacing the wild-
type 3'UTR of said naturally-encoded mitochondrially-
targeted mRNA, still allows for a mitochondrial target-
ing of the resulting mRNA.

[0237] It may be provided that, when said at least one MTS
nucleic acid sequence is the MTS RNA sequence of a natu-
rally-occurring nearly-encoded mitochondrially-targeted
mRNA, or the cDNA sequence of such a mRNA, or a DNA
sequence coding for such a MTS RNA sequence in accor-
dance with the universal genetic code, said at least one nucleic
acid CDS sequence is not the CDS of this naturally-occurring
nearly-encoded mitochondrially-targeted mRNA.

[0238] It may be provided that, when said at least one 3'
nucleic acid sequence is the 3'UTR sequence of a naturally-
occurring nearly-encoded mitochondrially-targeted
mRNA, or the cDNA sequence of such a mRNA, or a DNA
sequence coding for such a 3'UTR sequence, said at least one
CDS sequence is not the CDS of this naturally-occurring
nearly-encoded mitochondrially-targeted mRNA.

[0239] It may be provided that, when said at least one MTS
nucleic acid sequence and said 3' nucleic acid sequence,
respectively, are the MTS and 3'UTR sequences of a natu-
rally-occurring nearly-encoded mitochondrially-targeted
mRNA, or the cDNA sequences of such a mRNA, or a DNA
sequence coding for such a mRNA sequence, then said at least
one CDS sequence is not the CDS of this naturally-occurring
nearly-encoded mitochondrially-targeted mRNA.

[0240] Preferably, said nucleic acid construct does not
comprise any sequence which would be identical to:

[0241] the 3'UTR of a naturally-occurring mRNA which
is a nuclear-transcribed but not-mitochondrially-tar-
ggeted mRNA, or

[0242] the cDNA sequence of such a 3'UTR sequence, or

[0243] a DNA sequence coding for such a naturally-
occurring mRNA 3'UTR in accordance with the univer-
sal genetic code.

[0244] The resulting nucleic acid construct does not use a
post-translation importation pathway, but uses a co-transla-
tion importation pathway from nucleus to said mitochon-
drion.

[0245] The present invention more particularly relates to a
non-naturally occurring nucleic acid construct which com-
prises:

[0246] at least one mitochondrion-targeting nucleic acid
sequence (referred to as MTS nucleic acid sequence),

[0247] at least one nucleic acid sequence which encodes
said protein in accordance with the universal genetic
code (referred to as CDS sequence), and

[0248] at least one 3' nucleic acid sequence, which is
located in 3' of said at least one MTS nucleic acid
sequence and of said at least one CDS sequence,

[0249] wherein said at least one MTS nucleic acid sequence
is:

[0250] the cDNA sequence of the MTS RNA sequence of a
naturally-encoded mitochondrially-targeted mRNA,
or

[0251] a conservative variant or fragment of such a
cDNA sequence, which derives therefrom by deletion
and/or substitution and/or addition of one or several
nucleotides, but has retained a mitochondrion-targeting
function.

[0252] wherein said at least one 3' nucleic acid sequence is:

[0253] the cDNA sequence of the 3'UTR sequence of a
naturally-encoded mitochondrially-targeted mRNA, or

[0254] a conservative variant or fragment of such a
cDNA 3'UTR sequence, which derives therefrom by
deletion and/or substitution and/or addition of one or
several nucleotides, and which, when replacing the wild-
type 3'UTR of said naturally-encoded mitochondrially-
targeted mRNA, still allows for a mitochondrial target-
ing of the resulting mRNA.

[0255] Provided that, when said at least one MTS nucleic
acid sequence and said at least one 3' nucleic acid sequence,
respectively, are the MTS and 3'UTR sequences of a natu-
rally-occurring nearly-encoded mitochondrially-targeted
mRNA, or the cDNA sequences of such a mRNA, or a DNA
sequence coding for such a mRNA sequence, then said at least
one CDS sequence is not the CDS of this naturally-occurring
nearly-encoded mitochondrially-targeted mRNA, and

[0256] wherein said nucleic acid construct does not
comprise any sequence which would be identical to:

[0257] the 3'UTR of a naturally-occurring mRNA which
is a nuclear-transcribed but not-mitochondrially-tar-
ggeted mRNA, or

[0258] the cDNA sequence of such a 3'UTR sequence, or

[0259] a DNA sequence coding for such a naturally-
occurring mRNA 3'UTR in accordance with the univer-
sal genetic code.

[0260] Each and every feature, herein and above described
for the MTS, CDS, 3' nucleic acid sequences in relation with
the vector of the invention, and notably those features relating
to the MTS, CDS, 3' nucleic acid sequences, of course applies mutatis mutandis to the nucleic acid construct of the invention, and more particularly to the non-naturally occurring nucleic acid construct of the invention.

0261] Hence, it notably follows that:

0262] a MTS nucleic acid sequence of said nucleic acid construct can be the MTS nucleic acid sequence of ACO2, or of SOD2, or of ATP5b, or of UQCRFS1, or of NDUFV1, or of NDUFB1, or of COX10;

0263] a 3' nucleic acid sequence of said nucleic acid construct can be:

0264] the 3'UTR sequence of ACO2, or of SOD2, or of ATP5b, or of UQCRFS1, or of NDUFV1, or of NDUFB1, or of ALDH2, or of COX10, or of AK2, or

0265] the cDNA sequence of such a 3'UTR sequence, or

0266] a DNA sequence coding for such a 3'UTR sequence in accordance with the universal genetic code; and that

0267] illustrative nucleic acid constructs of the invention comprise or consist of a sequence of SEQ ID NO:21 (COX10 MTS-re-coded ATP6-COX10 3'UTR), and/or of SEQ ID NO:22 (SOD2 MTS-re-coded ATP6-SOD2 3'UTR), and/or of SEQ ID NO:25 (COX10 MTS-re-coded ND1-COX10 3'UTR), and/or of SEQ ID NO:26 (COX10 MTS-re-coded ND4-COX10 3'UTR).

0268] Said non-naturally occurring nucleic acid construct may be transfected in a cell in the form of naked DNA, or in the form of a plasmid. Any transfection technique which is found convenient by the skilled person is convenient. The skilled person may e.g. proceed by electroporation, DEAE Dextran transfection, calcium phosphate transfection, cationic liposome fusion, creation of an in vivo electrical field, DNA-coated microprojectile bombardment, ex vivo gene therapy, and the like.

0269] Said non-naturally occurring nucleic acid construct may of course alternatively and/or complementarily be inserted into a vector, such as a viral vector.

0270] The vector and nucleic acid construct of the invention are useful for nucleic acid therapy, e.g. to reverse a cellular dysfunction caused by a mutation in nucleic acid coding for a mitochondrially protein. They enable to restore a protein function in a cell.

0271] Engineered Cell:

0272] The present invention also relates to an engineered cell which has been transfected or infected by a vector according to the invention, and/or transfected by a nucleic acid construct according to the invention.

0273] Preferably, said engineered cell is an engineered animal or human cell, most preferably to a mammalian engineered cell, still more preferably to an engineered human cell.

0274] Said engineered cell may e.g. be a bone-marrow cell, a clonal cell, a germ-line cell, a post-mitotic cell, such as a cell of the central nervous system; a neuronal cell, a retinal ganglion cell, a progenitor cell; or a stem cell, a hematopoietic stem cell, a mesenchymal stem cell. Preferably, said engineered cell is a neuronal cell, a retinal ganglion cell.

0275] Said transduction, infection or transfection may be imprinted by any means available to the skilled person, e.g. by electroporation, DEAE Dextran transfection, calcium phosphate transfection, cationic liposome fusion, creation of an in vivo electrical field, DNA-coated microprojectile bombardment, injection with a recombinant replicative-defective virus, homologous recombination, ex vivo gene therapy, a viral vector, naked DNA transfer, and the like.

0276] Said engineered cell may e.g. be a cell, such as a neuronal cell, collected from a patient suffering from a disease related to a mitochondrial dysfunction. The vector and nucleic acid construct of the invention can indeed be used for ex vivo cell therapy.

0277] Pharmaceutical Compositions and Applications:

0278] The present invention also relates to a pharmaceutical composition comprising at least one vector according to the invention, or at least one nucleic acid construct according to the invention, or at least one engineered mammalian cell according to the invention.

0279] The present invention also relates to a drug comprising at least one vector according to the invention, or at least one nucleic acid construct according to the invention, or at least one engineered mammalian cell according to the invention.

0280] The compositions of the present invention may further comprise at least one pharmaceutically and/or physiologically acceptable vehicle (diluent, excipient, additive, pH adjuster, emulsifier or dispersing agent, preservative, surfactant, gelling agent, as well as buffering and other stabilizing and solubilizing agent, etc.).

0281] Appropriate pharmaceutically acceptable vehicles and formulations include all known pharmaceutically acceptable vehicles and formulations, such as those described in “Remington: The Science and Practice of Pharmacy”, 20th edition, Mack Publishing Co.; and “Pharmaceutical Dosage Forms and Drug Delivery Systems”, Ansel, Popovich and Allen Jr., Lippincott Williams and Wilkins.

0282] In general, the nature of the vehicle will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise, in addition to the one or more contrast agents, injectable fluids that include pharmaceutically and physiologically acceptable fluids, including water, physiological saline, balanced salt solutions, buffers, aqueous dextrose, glycerol, ethanol, sesame oil, combinations thereof, or the like as a vehicle. The medium also may contain conventional pharmaceutical adjunct materials such as, for example, pharmaceutically acceptable salts to adjust the osmotic pressure, buffers, preservatives and the like. The carrier and composition can be sterile, and the formulation suits the mode of administration.

0283] The composition can be e.g., be in the form of a liquid solution, suspension, emulsion, capsule, sustained release formulation, or powder.

0284] The pharmaceutical composition and drug of the invention are useful for the therapeutic and/or palliative and/ or preventive treatment of a disease, condition, or disorder related to a defect in activity or function of mitochondria.

0285] A mictomap is available on the MITOMAP website; this site notably provides with a list of mitochondrial disease-associated mutations.

0286] Scientific publication review relating to mitochondrial disease, condition, or disorder notably comprise Carelli et al. 2004 (Progress in Retinal and Eye Research 23: 53-89), DiMauro 2004 (Biochimica et Biophysica Acta 1659:107-114), Zeviani and Carelli 2003 (Curr Opin Neurol 16:585-594); and Schaefer et al. 2004 (Biochimica et Biophysica Acta 1659: 115-120), the contents of which being herein incorporated by reference.

0287] Diseases, conditions or disorders related to a defect in mitochondria activity or function notably comprise myo-
Optic neuropathies notably comprise:

- Leber’s hereditary optic neuropathy (LHON), which involves one or several point mutation(s) in mitochondrial DNA, more particularly point mutation(s) in the ND1 and/or ND4 and/or ND6 gene(s), such as G3460A (ND1 mutation), G11778A (ND4 mutation), T14484C (ND6 mutation).
- The dominant optic atrophy (DOA), also known as Kjer’s optic neuropathy, which involves a defect in nucleolin gene OPA1.
- The FHSN, MILS and NARP, which are the result of a mutation in the MTA1P6 gene (defective ATP synthesis), which can be corrected by restoring the activity of function of ATP6.

Leber’s hereditary optic neuropathy (LHON) was the first maternally inherited disease to be associated with point mutations in mitochondrial DNA and is now considered the most prevalent mitochondrial disorder. The pathology is characterized by selective loss of retinal ganglion cells leading to central vision loss and optic atrophy, prevalent in young males. It is a devastating disorder with the majority of patients showing no functional improvement and remaining within the legal requirement for blind registration. Other clinical abnormalities have also been reported in LHON patients. These include postural tremor, peripheral neuropathy, non-specific myopathy, movement disorders and cardiac arrhythmias [8]. The three most common pathogenic mutations from LHON affect complex I ND1 and/or ND4 and/or ND6 genes with the double effect of lowering ATP synthesis and increasing oxidative stress chronically.

Each of said disease, condition or disorder could be corrected by restoring activity or function of the mutated DNA.

Example 1 below illustrates the rescue of an ATP6 activity or function with a vector and nucleic acid of the invention. Example 2 below illustrates the rescue of a ND1 and ND4 activity or function with a vector and nucleic acid of the invention (fibroblasts collected from LHON patients).

The pharmaceutical compositions or drugs of the invention are more particularly intended for the therapeutic and/or palliative and/or preventive treatment of a myopathy or of an optic neuropathy, such as LHON, DOA, FHSN, MILS or NARP.

The present invention relates to the use of at least one vector or nucleic acid construct for in vivo or ex vivo therapy of a subject or patient in need of a therapeutic, palliative or preventive treatment of a disease, condition, or disorder related to a defect in activity or function of mitochondria.

The present invention also relates to the use of at least one vector or nucleic acid construct of the invention, or of at least one engineered cell of for the treatment of a disease, condition, or disorder related to a defect in activity or function of mitochondria, and more particularly for the production of a composition, pharmaceutical composition or drug intended for the treatment of such a disease condition, or disorder.

0298 The present invention more particularly relates to a method for the therapeutic and/or palliative and/or preventive treatment of a disease, condition, or disorder related to a defect in activity or function of mitochondria, which comprises:

- administering to a subject or patient in need thereof a vector and/or a nucleic acid construct and/or an engineered cell of the invention, in a quantity effective for the therapeutic and/or palliative and/or preventive treatment of said subject or patient,
- ex vivo treating cells collected from a subject or patient in need thereof, and
- returning the treated cells to the subject or patient.

0302 The term “comprising”, which is synonymous with “including” or “containing”, is open-ended, and does not exclude additional, unrecited element(s), ingredient(s) or method step(s), whereas the term “consisting of” is a closed term, which excludes any additional element, step, or ingredient which is not explicitly recited.

0303 The term “essentially consisting of” is a partially open term, which does not exclude additional, unrecited element(s), ingredient(s), method step(s), whereas the term “essentially consisting of” is a closed term, which excludes any additional element, step, or ingredient which is not explicitly recited.

0305 Each of the relevant disclosures of all references cited herein is specifically incorporated by reference.

0306 The present invention is illustrated by the following examples, which are given for illustrative purposes only.

EXAMPLES

Example 1

Allotopic Expression of the ATP6 Mitochondrial Gene is Significantly Improved by the Localization of its mRNAs to the Surface of Mitochondria Leading to an Efficient Import of the Precursor

0307 Abstract:

0308 It is clear that impairment of mitochondrial energy metabolism is the key pathogenic factor in a growing number of neurodegenerative disorders. With the discovery of mtDNA mutations, the replacement of defective genes became an important goal for mitochondrial geneticists worldwide. Unfortunately, before the present invention, it was still not possible to introduce foreign genes into the mitochondria of mammalian cells.

0309 To circumvent this problem, allotopic expression in the nucleus of genes encoded by mitochondrial DNA (mtDNA), became an attractive idea. However, for most mitochondrial genes tested, there were important limitations
related to the high hydrophobicity of the corresponding proteins, which impedes their mitochondrial translocation.

[0310] We herein elucidate the mechanisms that enable the delivery of mRNAs encoding mitochondrial proteins to the organelle surface, and demonstrate that this delivery depends on two sequences: the region coding for the mitochondrial targeting sequence (MTS) and the 3'UTR. mRNA sorting to mitochondrial surface permits to optimize allotypic approach, by enhancing the import efficiency of the precursor synthesized in the cytosol. As an illustration of this mechanism, we have chosen to utilize the sequence coding for the MTS and the 3'UTR of two nuclear genes encoding mitochondrial proteins: COX10 and SOD2 associated to a recoded mitochondrial ATP6 gene. Indeed, COX10 and SOD2 mRNAs localize to the mitochondrial surface in HeLa cells. HeLa cells transfected with these constructions express an ATP6 protein which is successfully delivered to the mitochondria. Hence, we have been able to optimize the allotypic approach for ATP6, and our procedure will next be tried to rescue mitochondrial dysfunction in patients presenting ATP6 mutations.

[0311] Introduction:

[0312] To examine the possibility that allotypic expression of mtDNA genes could be optimized by a targeted localization of the mRNA to the mitochondrial surface, we have chosen to utilize the sequences coding for the MTS and the 3'UTR of two nuclear genes encoding mitochondrial proteins: COX10 and SOD2 associated to a reengineered nucleus-localized ATP6 gene. COX10 encodes a highly hydrophobic protein of the inner mitochondrial membrane, its mRNAs localizes to the mitochondrial surface [5]. SOD2 encodes a mitochondrial protein involved in detoxification, its mRNA, as COX10 mRNA, localizes to the mitochondrial surface [5] and a recent report described that in HeLa cells, its 3'UTR is associated to the mitochondrial surface via the Atp121 protein [6]. The ability to synthesize and direct the ATP6 protein to mitochondria was examined in HeLa cells for 4 plasmids: two of them only contain the mts of COX10 or SOD2, and the other two possess both the MTS and the 3'UTR of each gene. Hybrid mRNAs were detected for each construction in both transiently and stably transfected cells. Further, ATP6 protein was also visualized by indirect immunofluorescence associated to the surface of mitochondria. Mitochondria isolated from transfected cells were examined for the presence of ATP6 protein. Remarkably, hybrids mRNAs possessing both the MTS and the 3'UTR of either COX10 and SOD2 allow the synthesis of a polypeptide which is imported in a highly efficient way from the cytosol into the mitochondria. Thus, the strategy of directing a hybrid mRNA to the mitochondrial surface significantly improves the feasibility of the allotypic approach for mitochondrial genes.

[0313] Material and Methods:

[0314] Plasmid Construction:

[0315] The full-length ATP6 mitochondrial gene was reengineered after the production of the 677 pb product by RT-PCR (Superscript III one step RT-PCR Platinum Taq HiFi, Invitrogen), using total RNA from HeLa cells. The PCR product obtained was cloned in the PCR 2.1-Topo vector (Invitrogen, Life technologies). In this vector, we recoded 11 non-universal codons in the ATP6 gene by four rounds of in vitro mutagenesis (Quik change Multi site-directed mutagenesis kit; Stratagene, La Jolla, Calif.). Six oligonucleotide primers were designed to alter AUG codons to UUG and UGA to UGG (Table 1).

<table>
<thead>
<tr>
<th>Name</th>
<th>sequence</th>
<th>Length</th>
<th>RT-PCR product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP6.1</td>
<td>CAATGGCTAATCAAACTAACCT</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>ATP6.2</td>
<td>CCTTATAGCTACTCCAAATGGTAC</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>ATP6.3</td>
<td>CCTACACTTTATATGGCAACATGCTCAC</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>ATP6.4</td>
<td>ACCCTATGGAATCC</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>ATP6.5</td>
<td>ACTAAAGGCAAGCACTGCTTC</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>ATP6.6</td>
<td>ACCACCACCTTTATTAATGCAACCTACCA</td>
<td>42</td>
<td></td>
</tr>
</tbody>
</table>

[0316] The intermediate construct was sequenced for accuracy. To this recoded ATP6, we appended in frame either the MTS of COX10 or SOD2, obtained by RT-PCR using total RNA from HeLa cells (Superscript III one step RT-PCR Platinum Taq HiFi, Invitrogen, Life technologies). For COX10 we amplified the sequence corresponding to the first 28 amino acids, for SOD2 the sequence coding for the first 30 amino acids. Oligonucleotide primers used for the amplification include at its 3' extremity a SalI restriction site for the subsequent cloning in frame with the reengineered ATP6 gene which possesses a SalI restriction site at its 5' extremity (Table 2).

<table>
<thead>
<tr>
<th>Name</th>
<th>5' Primer (5'-3')</th>
<th>3' Primer (5'-3')</th>
<th>RT-PCR product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP6</td>
<td>GTCGACCCCGCCA</td>
<td>CCCCGCGGCGGCGCTT</td>
<td>677</td>
</tr>
<tr>
<td>COX10</td>
<td>AGGAAATCTGTTTC</td>
<td>GCTTCACTCATT</td>
<td>(SEQ ID NO: 7) (SEQ ID NO: 8)</td>
</tr>
<tr>
<td>SOD2</td>
<td>CCGGTACCCGGA</td>
<td>ATACCGACGACGGC</td>
<td>TCC (SEQ ID NO: 9) (SEQ ID NO: 10)</td>
</tr>
<tr>
<td>O'UTR</td>
<td>CCCGTATCCCGGA</td>
<td>ATACAGACGACGGC</td>
<td>TCC (SEQ ID NO: 11) (SEQ ID NO: 12)</td>
</tr>
<tr>
<td>MTS</td>
<td>GGTGATGATGTT</td>
<td>GTCGACCGCCTGCG</td>
<td>90</td>
</tr>
<tr>
<td>SOD2</td>
<td>GGGCCGCGGATGT</td>
<td>GTCGACCGCCTGCG</td>
<td>215</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Name</th>
<th>5' Primer (5'-3')</th>
<th>3' Primer (5'-3')</th>
<th>RT-PCR product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX6c</td>
<td>ATGCTCTCCGAGAAGGG</td>
<td>CTTAAAGATTACACGCGG</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>(SEQ ID NO: 17)</td>
<td>(SEQ ID NO: 18)</td>
<td></td>
</tr>
<tr>
<td>SOD2</td>
<td>CCGAATGCGCCGCGG</td>
<td>CCGAATGCGCCGCGG</td>
<td>56</td>
</tr>
</tbody>
</table>

*indicates text missing or illegible when filed.

**TABLE 2 -continued**

Oligonucleotides primers for RT-PCR analysis

- Immunocytochemistry:
  - Coverslips were placed on the bottom of 24-well dishes and HeLa cells seeded at approximately 50% confluence (80000 cells). 60 hours after transfection, cells were fixed with 2% paraformaldehyde in PBS for 15 min and processed for indirect immunofluorescence. After permeabilization of the cells for 5 min with Triton X-1% in PBS, cells were incubated for one hour in PBS with 1% BSA before the addition of the primary antibodies: mouse monoclonal anti-Flag M2 antibodies (Stratagene, La Jolla Calif.) or mouse monoclonal anti-ATP synthase subunit beta (Molecular Probes, Invitrogen). Both antibodies were used at a final concentration of 1 microgram/ml. The incubation with primary antibodies was performed for either 2 hr at room temperature or overnight at 4°C. After washing the primary antibody three times five min with PBS, cells were incubated with the secondary antibody: labeled goat-anti-mouse IgG Alexa Fluor 488 (Molecular Probes, Invitrogen). This antibody was used in 1% BSA-PBS at 1:600 dilution and placed on the top of the coverslips for two hr. The cells were, subsequently, washed once in PBS for 5 min. For DNA and mitochondria staining, a second wash was performed with 0.3 microgrammes/ml of DAPI (Sigma, Saint Louis, Mich.) and 100 nM of MitoTracker Deep Red 633 (Molecular Probes, Invitrogen) for 20 min. A last 10 min wash was performed in PBS and the coverslips were mounted using Biomerica Gel Mount. Immunofluorescence was visualized in a Leica DM 5000 B Digital Microscope. Digital images were acquired and processed with the MetaVue imaging system software.

**[0323]** Mitochondria Isolation and Western Blot Analysis:

**[0324]** Between 20 to 40 millions or 100 millions of transiently or stably transfected HeLa cells were treated with trypsin (Gibco, Invitrogen) for 5 min and spin down. One wash in PBS was performed. The pellets were resuspended in 10 ml of homogenization buffer: 0.6 MmMn, 30 mM Tris-HCl pH 7.6, 5 mM MgAc and 100 mM KCl, 0.1% fatty acid-free bovine serum albumin (BSA), 5 mM beta-mercaptoethanol and 1 mM PMFS. To the resuspended cells 0.01% of digitonin was added. After a 4 min incubation on ice the homogenization was performed with 15 strokes in a Dounce glass homogenizer with a manually driven glass pestle type B. Homogenates are centrifuged for 8 min at 1000 g at 4°C to pellet unbroken cells and nuclei. Since many mitochondria remain trapped in this pellet, it was resuspended and rehomogenized again with 5 ml of homogenization buffer and 25 additional strokes. Then a second round of centrifugation under the same conditions was performed. Both supernatants were assembled and centrifuged again to discard any nuclear or cell contaminant. The supernatant obtained was centrifuged at 12000 g at 4°C for 30 min to pellet mitochondria. Four washes in homogenization buffer were performed to free the mitochondrial fraction of particles containing membranes, reticulum endoplasmic and proteases. The last two washes were performed in a homogenization buffer devoid of BSA and PMFS to allow a better estimation of the protein concentration in the final mitochondrial fraction and its subsequent analysis by proteinase K digestion. Protein concentrations in the extracts were measured using the dye-binding assay Bradford.

**[0325]** To determine whether Atp6 was translocated into the organelle, 15 microgrammes of mitochondrial proteins were treated with 200 microgrammes/ml of proteinase K (PK) at 0°C for 30 min. Samples were then resolved in 4-12 gradient or 12% polyacrylamide SDS-PAGE, and transferred
formed. The amount of precursor and mature forms of ATP6 in mitochondria, as well as the quantities of both the mature form of ATP6 and ATP, resistant to PK proteolysis were compared by densitometric analyses (Quantity One, Biorad software system). The significance of the differences observed was validated by a paired Student’s t-test.

[0327] RNA Extraction and RT-PCR Analyses:

[0328] Mitochondria extractions were performed as described in the preceding section, with the following modifications: 400 millions of cells were treated with 250 µg/ml cycloheximide for 20 minutes at 37°C. To HB was added 200 Mg/ml cycloheximide, 500 µg/ml heparine and 1/1000 RNAse inhibitor (rNasin, Promega). The last pellet of crude mitochondria associated with polysomes (M-P) was stored at -80°C C. until RNA extraction. Free-cytosolic polysomes (F-P) were obtained from the post-mitochondrial supernatant fraction by sedimentation through a step gradient of 2 M and 0.5 M sucrose. RNAs from these two fractions, as well as total RNAs from each stably transfected cell line, were obtained using RNeasy Protect Mini kit (Qiagen). Generally, 10 millions of cells are sufficient to obtain approximately 30 microgrammes of total RNA. The presence of the hybrid ATP6 mRNA was examined using primers which recognize the first 27 nt of either COX10 or SOD2 MTS and a primer which recognize the last 27 nt of the ATP6 ORF. For the pCMV-Tag 4A vector containing both the MTS and the 3'UTR of COX10 or SOD2, we used a primer recognizing the last 27 nt of each 3'UTR. 100 ng of RNA was used for reverse transcription (cf. Table 1 above). The products were then subjected to 25 cycles of PCR using Superscript III one step RT-PCR Platinum Taq kit (Invitrogen). As an internal control a 250 nt fragment within the ORF of COX6c gene, encoding a mitochondrial protein, was also amplified. Ten percent of the amplified products were run in agarose gels, and the quantities of amplified products reflecting hybrid ATP6 mRNA amount in each preparation was estimated using the Phototrap software (Vilber Lourmat; Torcy, France).

[0329] Tables 1 and 2 above, and table 9 below, show primer sequences, the expected sizes of the PCR products, the quantity of RNA used for reverse-transcription and the number of PCR cycles performed.

[0330] Densitometric analyses (Quantity One, Bio-Rad software) were performed the amount of both hybrid ATP6 and SOD2 transcripts in either mitochondrion-bound polysomes or free-cytosolic polysomes. Three independent RNA preparations from M-P and F-P fractions were subjected three times to RT-PCR analyses.

### Table 9

<table>
<thead>
<tr>
<th>mRNA</th>
<th>RT-PCR product</th>
<th>Primers</th>
<th>Total RNAs</th>
<th>Polyosomal RNAs (M-P/F-P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>length (bp)</td>
<td>5' Primer</td>
<td>3' Primer</td>
<td>Quantity (ng)</td>
</tr>
<tr>
<td>SOD2MTS</td>
<td>780</td>
<td>MTS</td>
<td>ATP6</td>
<td>200</td>
</tr>
<tr>
<td>ATP6</td>
<td>677</td>
<td>SOD2 5'</td>
<td>ORF3'</td>
<td>50</td>
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<tr>
<td>ATP6</td>
<td></td>
<td>ORF 5'</td>
<td>ATP6</td>
<td>100</td>
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<tr>
<td>SOD2</td>
<td>785</td>
<td>SOD2 5'</td>
<td>3'UTR</td>
<td>200</td>
</tr>
<tr>
<td>COX6c</td>
<td>250</td>
<td>COX6c 5'</td>
<td>COX6c 3'</td>
<td>200</td>
</tr>
</tbody>
</table>

[0331] Results:

[0332] Construction of Reengineered Mitochondrial ATP6 Gene for Allotopic Expression

[0333] To accomplish allotopic expression we synthesized the full-length version of nuclear-encoded ATP6 mitochondrial gene converting codons AUA to AUG and codons UGA to UGG. Indeed, AUA in the mitochondrial genetic system leads to the insertion of a methionine, but according to the universal code it is an isoleucine. Additionally, UGA into mitochondria codes for a tryptophan, whereas in the cytosol it represents a stop codon. We, therefore, recoded all 11 mitochondrial codons present in ATP6 ensuring the accurate translation of the transcript by cytoplasmic ribosomes. These alterations were performed by four rounds of in vitro mutagenesis using four independent oligonucleotide primers (Table 1) and the Quik change Multi site-directed mutagenesis kit (Stratagene, La Jolla, Calif.).

[0334] The concept of allotopic approach has important implications for the development of therapies to patients with mitochondrial DNA mutations. However, up today a major obstacle remains to be overcome and is the targeting of the recoded protein to mitochondria. We then decided to force the localization of the recoded ATP6 mRNA to the mitochondrial surface. The rationale behind this specific mRNA targeting is to allow a co-translational import mechanism which will maintain the precursor in an import competent conformation impeding its aggregation before or during translocation through the TOM (Translocase of the outer membrane) and TIM (Translocases of the inner membrane) import complexes. Two sequences within mRNAs are believed to be involved in their localization to the mitochondrial membrane: the sequence coding for the MTS and the 3'UTR. We have chosen two nuclearly-encoded mitochondrial genes, which
mRNAs are preferentially localized to the surface of mitochondria in HeLa cells: COX10 and SOD2 [5]. Interestingly, the SOD2 mRNA has been shown to be associated with the mitochondrial surface via its 3'UTR and the Akap121 protein. [0335] We therefore, obtained four different plasmids.

[0336] Two contain either the MTS of COX10 or the sequence encoding the first 30 amino acids of SOD2 (=the 20 amino acids of the MTS sequence of SOD2, and the ten consecutive amino acids that follows within the SOD2 sequence, i.e., fragment 1-30 of SEQ ID NO:49), in frame with the AUG codon of the recoded ATP6 gene (COX10 MTS-rcoded ATP6-SV40 3' UTR; SOD2 MTS-rcoded ATP6-SV40 3' UTR). In these plasmids, the SV40 polyA signal functions as the 3'UTR.

[0337] The other two combine both the MTS and the 3'UTR of COX10 and SOD2 respectively, and do not comprise the cytosolic 3'UTR of SV40 (COX10 MTS-rcoded ATP6-COX10 3' UTR; SOD2 MTS-rcoded ATP6-SOD2 3' UTR).

[0338] FIGS. 1A and 1B illustrate the constructs obtained and the full-length sequences inserted in the pcMV-Tag 4A vector than we named respectively: COX10 MTS-nATP6, SOD2 MTS-nATP6 and COX10 MTS-nATP6-COX10 3'UTR and SOD2 MTS-nATP6-SOD2 3'UTR.

[0339] Detection of Hybrid ATP6 mRNAs in Transiently and Stably HeLa Transfected Cells

[0340] To determine whether transfected cells express hybrid ATP6 mRNAs, steady-state levels of the transcripts were measured in both transiently and stably transfected cells after the isolation of total RNAs. 100 ng of total RNAs were subjected to RT-PCR analyses using specific primer oligonucleotides for hybrid ATP6 mRNA. COX10 gene encoding a mitochondrial protein was used as an internal control, with specific primers allowed the amplification of a 250 bp fragment (FIG. 2). RNAs from non-transfected HeLa cells as well as HeLa cells transfected with the empty pcMV-Tag 4A vector were also tested as negative controls. FIG. 2 shows a 780 bp PCR product corresponding to the amplification of the first 27 nt of COX10 ORF and the last 27 nt of the ATP6 ORF in cells transfected with both COX10 MTS-nATP6 and COX10 MTS-nATP6-COX10 3'UTR vectors. Additionally, RNAs isolated from cells transfected with the COX10 MTS-nATP6-COX10 3'UTR vector amplified a 2374 bp product corresponding to the entire ATP6 ORF and the full-length COX10 3'UTR.

[0341] The results obtained with RNAs purified from transfected cells with SOD2 MTS-nATP6 and SOD2 MTS-nATP6-SOD2 3'UTR vectors show that hybrid ATP6 transcript was detected as a 780 nt amplified product. Further, the SOD2 MTS-nATP6-SOD2 3'UTR region amplified a 1060 bp fragment, corresponding to the entire ATP6 ORF and the full-length SOD2 3'UTR. These results indicate that HeLa cells express the reengineered ATP6 gene. Moreover, no significant differences in the steady-state levels of hybrid mRNAs were found by the addition of either COX10 3'UTR or SOD2 3'UTR.

[0342] To examine the ability of SOD2 signals associated to the recoded ATP6 gene to direct hybrid mRNAs to the mitochondrial surface, we determined their subcellular localization in the four stably cell lines obtained. In this purpose, we isolated RNAs from mitochondrion-bound polysomes (M-P) and free-cytosolic polysomes (F-P) and we determined by RT-PCR the steady-state levels of hybrid mRNAs in both polysomal populations (FIG. 12B). As internal controls, the subcellular distribution of endogenous mitochondrial ATP6, SOD2 and COX6c mRNAs were determined. Endogenous ATP6 mRNA exclusively localized to the mitochondrial compartment as expected. Besides, endogenous SOD2 mRNA is enriched in mitochondrion-bound polysomes (M-P), whereas COX6c mRNA is preferentially detected in free-cytosolic polysomes (F-P) as we have previously observed. The SOD2MTSATP6-3'UTR-SOD2 vector directed the synthesis of a hybrid mRNA that was almost undetectable in free-cytosolic polysomes. Hybrid mRNA produced from the SOD2MTSATP6-3'UTRSV40 plasmid was also detected preferentially in mitochondrion-bound polysomes. However, it was also present in free-cytosolic polysomes (FIG. 12B). Densitometric analyses were performed to determine the amount of both endogenous SOD2 and hybrid ATP6 mRNAs in each polysomal population examined. SOD2 mRNA signal in mitochondrion-bound polysomes was 85.6%±6.15 in cell lines expressing the SOD2MTSATP6-3'UTRSV40 plasmid and 82.5%±4.87 in cells expressing the SOD2MTSATP6-3'UTR-SOD2 vector. Interestingly, it was found for the ATP6 hybrid mRNA that only 72.4%±5.2 localized to the mitochondrial surface in cells expressing the SOD2MTSATP6-3'UTRSV40 vector. Instead, in cells expressing the SOD2MTSATP6-3'UTR-SOD2 vector 84.6%±4.7 of the hybrid mRNA localized to the mitochondrial surface (FIG. 12C). These values were significantly different according to the paired Student’s t-test (P<0.0034, n=6). Thus, the combination of both the MTS and 3'UTR of SOD2 to the reengineered ATP6 gene leads to the synthesis in the nucleus of a transcript which was almost exclusively sorted to the mitochondrial surface. Indeed, its subcellular distribution is not significantly different to the one of the endogenous SOD2 mRNA.

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[0343] Detection of ATP6 Allotopic Expression in HeLa Cells by Indirect Immunofluorescence

[0344] We analyzed the ability of the reengineered ATP6 product to localize to mitochondria in vivo.

[0345] For this, we appended a Flag epitope in frame to the C-terminus of the ATP6 ORF and we examined stably transfected cells by indirect immunofluorescence (FIG. 13). HeLa cells transfected with the empty pcMV-Tag 4A vector were used as negative controls and showed a low diffused signal in cytoplasm when antibodies to Flag were used (FIG. 13, left panel). Stably transfected cells with either SOD2MTSATP6-3'UTR Flag or SOD2MTSATP6-3'UTR Flag vectors were visualized by indirect immunofluorescence using antibodies to Flag (FIG. 14, left panel) and to ATP synthase subunit α (FIG. 13 middle panel). For each cell type visualized, a merged image in association with DAPI staining is shown in the right panel. A typical punctuate mitochondrial pattern was observed in cells expressing the recoded ATP6 polypeptides, when the Flag antibody was used. This indicates that fusion ATP6 proteins localized to mitochondria.
[0346] Immunocytochemistry to detect the flag epitope in HeLa cells transiently or stably transfected with the four pCMV-Tag 4A vectors showed a typical punctate mitochondrial pattern, suggesting that the fusion Atp6 protein had been localized within the mitochondria (Fig. 3). Indeed, this typical punctuate mitochondrial pattern was also observed using either the mitochondrion-specific dye Mito Tracker Red or specific antibodies anti-ATP synthase subunit beta. HeLa cells transfected with the empty pCMV-Tag 4A vector were used as negative controls and showed a diffuse cytoplasmic distribution but with a low intensity (Fig. 3). The localization patterns of the different Atp6 peptides which synthesis were directed by the four pCMV-Tag 4A vectors were essentially identical confirming that both COX10 and SOD2 sequences successfully allowed the reengineered Atp6 protein to localize to the mitochondria in vivo.

[0347] Translocation of the Fusion Atp6 Protein into Mitochondria of HeLa Cells

[0348] To determine whether reengineered Atp6 gene products are efficiently imported into mitochondria in vivo, mitochondria isolated from stably transfected HeLa cells were subjected to western blot analysis (Fig. 4). We visualized two forms with anti-flag antibodies of approximately 30 and 20 kDa, representing the precursor and mature forms of the recombed Atp6 protein.

[0349] The predicted molecular weights of both proteins are respectively 34 and 30 kDa, larger than the ones implied by the molecular weight markers. This discrepancy has often been observed when extremely hydrophobic proteins were migrated in SDS-PAGE. In general, the electrophoretic mobility on SDS-PAGE of proteins encoded by mtDNA is higher than the one expected for their theoretical molecular weights.

[0350] The steady-state levels of both polypeptides are similar in the two cell lines examined: cells transfected with MTS COX10-nAtP6 vector (MTS COX10-nAtP6), and cells transfected with MTS COX10-nAtP6-COX10 3'UTR (MTS COX10-nAtP6-3'UTR).

[0351] To determine the amounts of the recombed Atp6 polypeptides produced in HeLa cells expressing either SOD2MTS AtP6-3'UTR3'UTR or SOD2MTS AtP6-3'UTR SOD2 vectors, we compared six independent mitochondrial extractions (Fig. 14A). Both precursor and mature polypeptides were equally abundant in mitochondria from each cell line, indeed the expression of SOD2MTS AtP6-3'UTR3'UTR vector leads to an accumulation of 61.4% of the precursor form. Instead, SOD2MTS AtP6-3'UTR SOD2 vector directed the synthesis of 64.4% of the precursor. These values were not significantly different according to the paired Student’s t-test. Similar results were obtained when total extracts from each cell line were examined by Western blotting. This data is in agreement with the over all amounts of hybrid AtP6 mRNAs detected when total RNAs from cell lines expressing either SOD2MTS AtP6-3'UTR3'UTR or SOD2MTS AtP6-3'UTR SOD2 vectors were subjected to RT-PCR analyses (Fig. 12A).

Therefore, the steady-state levels of the AtP6 precursor and its ability to recognize the TOM complex in the outer mitochondrial membrane do not depend on the presence of the SOD2 3'UTR. Notably, the relative proportions of AtP6 precursor and mature forms were analogous to the ones shown in cells for highly hydrophobic proteins en route to the mitochondrial matrix.

[0352] When mitochondria were treated with 150 or 200 microgrammes/ml of proteinase K (PK) the precursor forms of the fusion ATP6 protein were sensitive to proteolysis in both cell lines. In contrast the mature form of AtP6 is resistant to PK digestion, especially in cells expressing the MTS COX10-nAtP6-COX10 3'UTR transcript. Indeed, in these cells the amount of the mature ATP6 protein is approximately 185% higher than in cells expressing the MTS COX10-nAtP6 mRNA. These data strongly indicates that not only the precursor polypeptide is correctly addressed to the surface of mitochondria, as we observed by indirect immunofluorescence (Fig. 3), but also that it was efficiently translocated into the organelle and correctly processed. Moreover, Fig. 4 shows that the quantity of the mature form of the ATP6 protein and the 65 kDa ATPalpha protein inside the mitochondria were quite similar after proteinase K digestion. Therefore, the use of COX10 MTS allows an efficient mitochondrial translocation of the recombed ATP6 protein, and when COX10 MTS is combined to the 3'UTR of COX10 a significant more efficient in vivo translation/import of the alloptically expressed ATP6 gene is obtained.

[0353] FIG. 14B upper panel shows a schematic representation of the theoretically expected ATP6 import intermediate. The hydrophobic passenger ATP6 precursor can be trapped en route to the matrix and a mitochondrial processing peptidase can cleave the MTS. Nevertheless, the rest of the protein remained accessible to PK action and therefore becoming undetectable on Western blotting. Instead, the fraction of the ATP6 protein which can be completely translocated is insensitive to PK-induced proteolysis and can therefore be integrated into the inner mitochondrial membrane, hence, remaining detectable on immunoblotting.

[0354] FIG. 14B shows that precursor forms of the fusion protein were sensitive to proteolysis in both cell line examined. Nearly all the ATP6 precursor signal disappeared after PK digestion, so precursors that were engaged in the process of translocation or loosely attached to the outer mitochondrial membrane but not fully translocated, were entirely digested (FIG. 14B, middle panel). In contrast, a significant amount of the mature form of ATP6 is resistant to PK digestion, indicating its location inside the organelle. To examine the levels of another complex V protein in these cells, immunoblotting were performed using anti-ATP synthase α antibody. This naturally imported mitochondrial protein was present at similar extents in all cells tested. Only one band of approximately 65 kDa was visualized suggesting that either we were unable to discriminate the precursor and mature forms of this protein under the electrophoretic conditions used or precursor polypeptides were very rapidly and efficiently translocated. Additionally, no major differences of the ATP synthase α signals were detected after PK treatment, confirming the integrity of the mitochondrial isolations (FIG. 14B, middle panel). To compare the import efficiency of the recombed ATP6 proteins in cells transfected with either SOD2MTS AtP6-3'UTR3'UTR or SOD2MTS AtP6-3'UTR3'UTR SOD2 vectors, we measured the amount of the mature form of ATP6 insensitive to PK digestion in each cell line, after normalization with the amount of ATP synthase α resistant to PK proteolysis. Results for six independent mitochondrial extractions subjected to immunoblotting analyses were shown in Fig. 15B, lower panel. Overall results show that both SOD2 signals lead to a high efficient import of the recombed ATP6 precursor. Remarkably, the level of the mature form insensitive to PK proteolysis in cells transfected with SOD2MTS AtP6-3'UTR3'UTR (1.28±0.24) was 1.8 fold higher than in cells expressing the SOD2MTS AtP6-3'UTR3'UTR (0.71±0.12).
sured was significant according to the paired Student’s t-test (P<0.0022, n=6). This observation could be related to the higher enrichment in the mitochondrion-bound polysomes of the corresponding mRNA (FIG. 12B).

To test whether reengineered ATP6 proteins were assembled into the ATP synthase complex. The complex is organized in F0-F1 domains. The F1 sector is a water-soluble unit located in the matrix and having the ability to hydrolyze ATP. The F0 domain is embedded in the inner membrane and is composed of hydrophobic subunits forming a proton pathway. ATP6 is an intrinsic protein of F0, composed of five putative transmembrane α-helices. In contrast, ATP synthase α is located in the matrix F1 domain. Studies performed with bovine heart mitochondria demonstrated that ATP6 was degraded at a very low rate when F0 subunits were subjected to trypsin treatment. Therefore, we treated mitochondria with both PK and Triton X-100 (1%). The detergent disrupts both mitochondrial membranes and theoretically leads to the entire proteolysis of mitochondrial proteins, demonstrating their localization somewhere inside the organelle in a protease-sensitive form. FIG. 3C shows that indeed ATP synthase α was fully digested by PK; instead, a significant amount of ATP6 remained insensitive to PK proteolysis. This result suggests that the recombined ATP6 was assembled into complex V.

Discussion:

Recent, epidemiological studies demonstrated that as a group, disorders of the mitochondrial function affect at least 1 in 5000 of the population, making them among the most common genetically determined disorders. In spite of the fact that over the last decade, the underlying genetic bases of several mitochondrial diseases involving central nervous system degeneration, no effective therapy is available for mitochondrial disorders. Pathogenic point mutations of genes encoded by the mitochondrial genome have been described as the cause of many mitochondrial disorders. A possible therapeutic approach is to exploit the natural mitochondrial protein import pathway. The basic concept is to introduce a wild-type copy of the mutated mitochondrial gene into the nucleus and import normal copies of the gene product into mitochondria from cytosol. This concept has been termed allotypic expression and several reports in yeast described that a number of non mitochondrial polypeptides can be relocated to the mitochondrial matrix simply by conjugating a targeting sequence to their N-terminus. However, when this approach has been tried in mammalian cells using different MTS and genes encoded by mtDNA, precursors were not imported efficiently into mitochondria. By contrast, the rescue of mitochondrial defect in patient cells was not only partial but also temporary [7]. Therefore, up today the spectrum of mtDNA encoded polypeptides than can be successfully expressed and integrated into mitochondrial respiratory chain complexes is very limited. This limitation is thought to be the consequence of the high hydrophobicity nature of mtDNA encoded proteins, which possess transmembrane domains refractive to mitochondrial import. The precursor synthesized in the cytosol could lack the import-competent structure required for an efficient mitochondrial membrane translocation.

The concept of mesohydrophobicity is likely to be an important factor for mitochondrial import competency. Mesohydrophobicity describes the average hydrophobicity in a window of 60-80 amino acids, together with the calculation of the most hydrophobic 17-amino acid segment. This calculation could predict importability of hydrophobic peptides. Using their algorithm, we analyzed this correlation to assess the mitochondrial importability of SOD2αMT6 ATP6 gene product and compared to ATP6, COX8 and SOD2 polypeptides as well as the previously tested fusion protein COX8αMT6 ATP6: as the wild-type ATP6, both fusion proteins examined cannot be translocated into mitochondria, mainly due to the high hydrophobicity of ATP6. Hence, a possibility that can allow the import of a recombined ATP6 protein into the organelle is that the precursor is engaged in a co-translational pathway of import. Thereby, the precursor would be maintained in a loosely folded nonaggregated conformation required for translocation through the mitochondrial import apparatus.

To overcome this limitation and try to develop a more long-term and definitive rescue of mtDNA mutations by allotypic expression leading to its application in gene therapy, we decided to construct nuclear versions of the mtDNA encoded ATP6 gene in which we appended the signals intended for forcing the hybrid mRNA to localize to the mitochondrial surface. We have chosen COX10 and SOD2 genes, which transcripts are enriched in the mitochondrial surface.

We were able to demonstrate that the association to a recombined ATP6 gene of both the MTS and 3'UTR signals leading to a mRNA delivery to the mitochondrial surface unambiguously improves the feasibility of the allotypic approach for mitochondrial genes. Indeed, not only we were able to visualize the protein in the mitochondria by indirect immunofluorescence, but most important the amounts of the processed Atp6 polypeptide inside the organelle were quite similar to the naturally imported ATPalpha protein. This result strongly indicates that the recombined ATP6 precursor was efficiently imported, the improvement we were able to produce as compared to other recent reports [1], [2], [3], [4] is certainly due to the fact that the hybrid mRNA was addressed to the mitochondrial surface, therefore enhancing the coupling between translation and mitochondrial import processes.

Most interestingly, we obtained a gradual improvement, indeed the use of either COX10 or SOD2 MTS alone, gave a good result in which approximately 50% of the mature ATP6 protein is translocated inside the mitochondria. When each MTS was combined to the corresponding 3'UTR, at least 85% of the mature ATP6 protein is insensitive to proteinase K digestion indicating that almost all the protein synthesized in the cytoplasm is successfully translocated inside the mitochondria.

To accomplish allotypic expression, the localization of an mRNA to the mitochondrial surface has never been tried before. In the allotypic approaches reported, even though different MTS were appended to recombined mitochondrial genes, all the constructs examined contained at their 3'-extremities the SV40 polYA signal that does not lead to any specific subcellular localization of the transcript. Our data clearly demonstrate that the association to a recombined ATP6 gene of both the MTS and 3'UTR signals of the SOD2 gene leads to a high efficient delivery of the hybrid mRNA to the mitochondrial surface. This improves unambiguously the feasibility of the allotypic approach for this mitochondrial gene. Indeed, not only were we able to visualize ATP6 protein in the mitochondria by indirect immunofluorescence but definitely the amount of the processed ATP6 polypeptide inside the organelle was quite similar to the naturally imported ATP
synthase subunit α, a Complex V component, such as is ATP6. These data strongly indicate that reencoded ATP6 precursors were successfully imported. The improvement we obtained compared to a recent report, which measured 18.5% of the precursor translated, is certainly due to the localization of hybrid mRNAs to the mitochondrial surface. This specific localization obviously enhances the coupling between translation and import processes, therefore, diminishing the block of the precursor during its translocation through the TOM and TIM complexes. It is worth mentioning that we obtained a gradual improvement on mitochondrial import of the ATP6 precursor. When both the MTS and the 3’UTR of SOD2 were combined, the amount of fully translocated ATP6 protein was 1.8-fold higher than when just the MTS was present. This is likely related to the improvement of mRNA sorting to the mitochondrial surface when both cis-acting elements of SOD2 were associated to the reencoded ATP6 gene. Remarkably, proteolysis insensitivity of the translocated ATP6 protein in the presence of both PK and Triton X-100 suggested that the protein could be correctly assembled in the F0 domain of the respiratory chain Complex V. Notably, combining the cis-acting elements of the COX10 gene to the reencoded ATP6 gene, we obtained an effective mitochondrial import ability of the fusion protein. Indeed, COX10 mRNA codes for a highly hydrophobic protein involved in Complex IV biogenesis, and SOD2 mRNA is enriched at the mitochondrial surface in human cells.

We clearly demonstrated that the association to a reencoded ATP6 gene of both the MTS and 3’UTR signals of either SOD2 or COX10 genes leads to a high efficiency delivery of hybrid ATP6 mRNAs to the mitochondrial surface, especially when both the MTS and the 3’UTR of SOD2 or COX10 were associated to the reengineered ATP6 gene. This specific subcellular localization of hybrid mRNAs leads to a high efficiency in the mitochondrial translocation of the reencoded ATP6 proteins. Remarkably, when both the MTS and the 3’UTR of either SOD2 or COX10 were combined, the amount of fully translocated ATP6 protein was 1.8-fold higher than when the MTS was associated to the cytosolic SV40 3’UTR. Therefore, the improvement of mRNA sorting to the mitochondrial surface when both cis-acting elements of SOD2 or COX10 were associated to the reencoded ATP6 gene definitely increase the amount of the processed ATP6 polypeptide inside the organelle which became quite similar to the naturally imported ATP synthase subunit α, a Complex V component, such as is ATP6. Thus, by directing a hybrid mRNA to the mitochondrial surface we significantly improve the feasibility of the allelic approach for the ATP6 mitochondrial gene.

In conclusion, we optimize the allotypic expression approach for ATP6, by the use of mRNA targeting signals without any amino acid change in the protein that could affect biologic activity.

This approach becomes henceforth available to rescue mitochondrial deficiencies caused by mutations in mtDNA genes.

Example 2
Correct Mitochondrial Localization of the Recoded Mitochondrial ND1 and ND4 Genes in Fibroblasts from LHON Patients

The three most common pathogenic mutations from LHON affect complex I ND1, ND4 and ND6 genes with the double effect of lowering ATP synthesis and increasing oxidative stress chronically.

Since we have demonstrated that reengineered mitochondrial Atp6 proteins were successfully translocated inside the mitochondria in HeLa cells (see example 1 above), we decided to synthesize reencoded mitochondrial genes ND1 and ND4. To ensure the efficient import of the allotypically expressed proteins we appended to them signals which will direct the corresponding mRNAs to the mitochondrial surface. We have chosen to use the MTS of COX10 gene alone or in combination with its entire 3’UTR. FIGS. 5A and 5B illustrate the constructs obtained and the full-length sequences inserted in the pCMV-Tag 4A vector.

Material and Methods:

Cell Culture and Transfection:

Fibroblasts were obtained from LHON patients of the Hôpital Necker Enfants Malades, Paris, France (Département de Génétique). We cultured these cells with D-MEM medium supplemented with 10% of foetal bovine serum, pyruvate, gentamicin (0.01%), and 2 mM glutamine. When indicated cells were grown in glucose-free medium supplemented with 10 mM galactose.

Fibroblasts were transfected with FoGENE 6 transfection reagent as recommended by the manufacturer (Roche Biochemicals, Indianapolis). Briefly, monolayer fibroblast cells were seeded a day before transfection at 50% confluence, so the next day they will be at approximately 80% confluence, the cells were plated in a medium without antibiotics. 2 microgrammes of different plasmids purified with Quiagen plasmid midi kit (Quiagen; Valencia, Calif.) were used. Between 48 to 60 hr later, 80% of the transfected cells were used for immunocytochemistry analyses. The remaining 20% of cells were selected for neomycin, G418, resistance (selectable marker present in the pCMV-Tag 4A vector) at a final concentration of 0.25 mg/ml. Stable clones were expanded for several weeks.

Optimized Recoding into Human Genetic Code

mtDNA has been reencoded according to human genetic code, taking into account the preferred codon usage in human:

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<th>TABLE 3</th>
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% GC 63/42

[0379] Detection of ND4 Allotypic Expression in Fibroblasts from LHON Patients Presenting the G11778A ND4 Mutation

[0380] Two plasmids directing the synthesis in the cytosol of a recoded wild-type ND4 gene were obtained. One of them, COX10 MTs-nND4-SV40 3' UTR, possesses appended to the N-terminus of the protein the sequence corresponding to the first 28 amino acids of COX10. The second one, COX10 MTs-nND4-COX10 3' UTR, has in addition at the end of the ORF the full-length 3' UTR of COX10. Fibroblasts from a patient presenting 100% of mtDNA molecules with the G11778A ND4 mutation were transiently transfected with either one of these plasmids. 60 h after cells were fixed and visualized to determine the ability of the COX10 sequences to target the recoded protein to the mitochondria. FIG. 7 shows that in both cases the fusion MTs COX10ND4Flag protein did have a punctate staining pattern, which is very similar to the one observed for the same cells with the naturally imported mitochondrial protein ATP synthase subunit beta. Thus, implying that the recoded ND4 fusion protein was imported into mitochondria.

[0381] In conclusion, as for the mitochondrial ATP6 gene, we were able to optimize the allotypic expression approach for ND1 and ND4 genes, by the simply use of mRNA targeting signals without any amino acid change in the protein that could affect biologic activity.

[0382] Growth Ability of LHON Fibroblasts in Galactose Medium

[0383] Fibroblasts presenting the G3460A ND1 mutation were grown with galactose, which slowly enters glycolysis as compared to glucose. FIG. 8 shows major differences in cell growth after six day culture: fibroblasts presented a severe growth defect, less that 10% of the cells survived in medium containing galactose as compared to cells seeded in glucose-rich medium. Stably transfected fibroblasts with the MTs COX10-nND1-COX10 3' UTR vector had a markedly improved rate of growth in galactose compared with that of non-transfected cells. This result implies that the mitochondrially imported recoded ND1 protein had assembled successfully into functional complex I allowing, therefore, a rescue of mitochondrial dysfunction in these cells.

Example 3

Rescue of Mitochondrial Deficiency Causing Human Diseases (Transfection of Fibroblasts from a NARP Patient)

[0384] We also determined whether the reengineered ATP6 protein would be able to rescue mitochondrial deficiency in cells having a mutated ATP6 gene.

[0385] We obtained fibroblasts from a patient presenting NARP disease caused by the T8993G mutation in the ATP6 gene.

[0386] Fibroblasts were cultured on media containing sodium pyruvate and relatively high amounts of FBS, more particularly:

[0387] on a medium containing glucose (D-MEM with L-glutamine, 4500 mg/L D-glucose, 110 mg/L sodium pyruvate 2.5 mM, FBS 15%, uridine 28 microM), or
on a medium, which does not contain glucose, but contain galactose (liquid D-MEM (1x), with L-Glutamine without Glucose, sodium pyruvate 2.5 mM, galactose 10 mM, FBS 15%, uridine 28 microM).

Stably transfected cells expressing the nuclear version of ATP6 associated with either SOD2 MTS alone, or in combination with SOD2 3'UTR, were obtained. Respiratory chain activity has been examined by the ability of these cells to grow in a medium in which glucose has been replaced by galactose for either 10 or 20 days. NARP cells expressing the empty vector had a low survival rate (30%). Cells expressing ATP6 with either the MTS of SOD2 or both the MTS and the 3'UTR of SOD2 present a growth survival of approximately 60%. If the selection was maintained for 20 days, the survival growth rate of cells expressing our optimized vectors was superior to 80% (FIG. 16). Only subtle differences of survival rate were observed for fibroblasts expressing either the vector with both the MTS and 3'UTR of the SOD2 gene or the vector with the SOD2 MTS associated to the SV40 3'UTR. This, is certainly due to the fact that these cells are heteroplasmic for the T8993G mutation, indeed they possess approximately 10% of the wild-type gene. Therefore, in their mitochondria probably 10% of a functional ATP6 protein could be assembled in Complex V. We can envision that the expression of the vector with SOD2 MTS associated to the SV40 3'UTR, will lead to the mitochondrial import of enough ATP6 protein to allow the cells to growth a good rate in galactose medium.

Preliminary measures of the real amount of ATP produced in vitro by fibroblasts expressing either one of our vectors clearly show a difference in the activity of Complex V related to the presence of either SOD2 3'UTR or SV40 3'UTR. Hence, when compared to control fibroblasts (100% of ATP synthesis in galactose medium) NARP fibroblasts expressing the vector with SOD2 MTS associated to the SV40 3'UTR had 50%, representing an increase compared to non transfected NARP cells (30%) but was less important when compared to the amount found in cells expressing the vector which combines to the transduced ATP6 gene both the MTS and the 3'UTR of the SOD2 gene (approximately 85%); cf. FIG. 19. By consequence, a more complete and efficient rescue of mitochondrial dysfunction is obtained when allotopic approach implies the presence of both the MTS and 3'UTR targeting signals.

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<th>Fibroblasts</th>
<th>Survival rate on galactose</th>
<th>Rate of ATP synthesis on galactose</th>
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<tr>
<td>Control</td>
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Example 4 Rescue of Mitochondrial Deficiency Causing Human Diseases (Transfection of Fibroblasts from LHON Patients)

The applicability potential of the improved allotopic expression approach of the inventors has been further confirmed by examining two other mtDNA genes involved in LHON. The fibroblasts obtained presented a total homoplasy of the mutation; indeed all the molecules of mitochondrial DNA are mutated.

Fibroblasts were cultured on media containing sodium pyruvate and relatively high amounts of FBS, more particularly:

- [0393] on a medium containing glucose (D-MEM with L-glutamine, 4500 mg/L D-glucose, 110 mg/L sodium pyruvate 2.5 mM, FBS 15%, uridine 28 microM), or
- [0394] on a medium, which does not contain glucose, but contain galactose (liquid D-MEM (1x), with L-Glutamine without Glucose, sodium pyruvate 2.5 mM, galactose 10 mM, FBS 15%, uridine 28 microM).

The engineered nucleus-localized versions of ND1 and ND4 were obtained; ND1 and ND4 transcripts possess both at their 5' and 3' extremities COX10 mRNA targeting sequences. Stable transfections of these constructions in fibroblasts from LHON's patients with either ND1 or ND4 mutations were performed. Indirect immunofluorescence showed that both proteins localize to the surface of mitochondria in vivo. The OXPHOS activity of these cells has been also examined by growing in a galactose rich medium. Interestingly, fibroblast cells allotopically expressing the wild-type ND4 protein showed a markedly improved rate of growth on galactose medium. This improvement is higher when both the MTS and 3'UTR of COX10 were associated to the ND4 gene (54.2%) as compared to that of mock transfected cells (8%) or to the cells transfected with the ND4 gene associated to the MTS of COX10 and the cytosolic SV40 3'UTR (12.7%) (FIG. 17). MTS: ND4 associated to COX10 MTS and the SV40 3'UTR, 3'UTR: ND4 associated to both the MTS and 3'UTR of COX10. This data imply that in spite of the presence in these cells of the ND4 mutated polypeptide, the wild-type protein was successfully imported into the organelle and assembled in Complex I. Preliminary experiments, of in vitro measurements of ATP synthesis confirm these results, indeed in untransfected cells very little ATP was synthesized in galactose medium (14% of the control level measured in healthy fibroblasts), when cells express the ND4 gene associated to the MTS of COX10 and the cytosolic SV40 3'UTR an increased is observed (56%). Remarkably, this increase is more important when cells express the ND4 gene associated to both the MTS and 3'UTR of COX10 (84%).

<table>
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<th>Rate of ATP synthesis on galactose</th>
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<td>LHON + mitochondrial 3'UTR (COX10 3'UTR)</td>
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Example 5

Transduction of Retinal Ganglion Cells

The inventors obtained, by in vitro mutagenesis, reengineered ND1, ND4, ND6 and ATP6 genes, which possess the most common mutations found in LHON's and NARP's patients: G3460A, G11778A, T14484C and T8993G respectively. Both wild-type and mutated genes have been integrated in the p-AAV-ires-hrGFP vector, which will allow the production of infectious recombinant human Adeno-Associated Virus Type 2 (AAV2) virions. For all constructions, each nuclear version of mtDNA genes is associated to the two mRNA targeting sequences of the COX10 gene, which allow the enrichment of corresponding mRNAs at the surface of mitochondria.

In accordance with the present invention, this will ensure the efficient delivery of the polypeptides inside the organelle.

Retinal ganglion cells (RGC) represent the primary cellular target of the pathogenic process of LHON disease.

The inventors purified RGCs from adult rat retina, thereby obtaining enriched RGC populations, and maintained them in culture for two weeks. Mitochondria are distributed along actin filaments and they specifically concentrated at the extremities of neuron extensions. The inventors transfected these cells with the mutated version of the ND1 gene. Preliminary results showed that the expression at high levels of the mutated protein during 8 days leads to an abnormal distribution of mitochondria along the neurite and cone extensions (FIG. 18).

BIBLIOGRAPHIC REFERENCES CITED IN THE EXAMPLES


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gcttcccgct tgcggccagc aggagctgg gttggggtg ggggttcctta aaaaatcttt 180
ctttggggtac gccttgctat tttggttttg gttcagatct taagccagctc atgaactctg 240
tatttttttttt tcagagcaca acotccatctt aaagttttttt tccgcttgta ccattttctt 300
gggccggcat ggtcctgttgaa cagccctttt tctctggaag gaaacctcag atctctctttt 360
agaaaaa aaaaaaaaaa aa 382

<210> SEQ ID NO 34
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 34
Met Leu Ser Arg Ala Val Cys Gly Thr Ser Arg Gln Leu Ala Pro Ala
1 5 10 15
Leu Gly Tyr Leu
20
<210> SEQ ID NO 35
<211> LENGTH: 214
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 35
accaagatcg ttatgctgt catacctaa tgatcccagc aagataaagt cctgttctct 60
aagatgtgca ctaagctcctg tacatactga aaccctata aggtctcgga taatttttgt 120
ttgatatttc atggaagaa cattatatct ccaatgttg gaagtttttg actgttaata 180
aagataactgt ctaaccatca aaaaaaaaaa aaaa 214

<210> SEQ ID NO 36
<211> LENGTH: 63
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 36
Met Leu Gly Phe Val Gly Val Ala Ala Ala Ala Pro Ala Ser Gly Ala
1  5  10  15
Leu Arg Arg Leu Thr Pro Ser Ala Ser Leu Pro Pro Ala Gln Leu Leu
20 25 30
Leu Arg Ala Ala Pro Thr Ala Val His Pro Val Arg Aep Tyr Ala Ala
35 40 45
Gln Thr Ser Pro Ser Pro Lys Ala Gly Ala Thr Gly Arg Ile
50 55 60

<210> SEQ ID NO 37
<211> LENGTH: 162
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 37
gggctcttgg tcctcgtacc tgtctctcct ccctgccccta aaccaaaaag cttcatttttt 60
cctgtgagcc tgcacagac ccttgctagc aatgataaca cttctgtcagc gatatttaaag 120
tttcctataa aatgtaaccc cttccagaaaa aaaaaaaaaa aas 162

<210> SEQ ID NO 38
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 38
Met Leu Ser Val Ala Ala Arg Ser Gly Pro Phe Ala Pro Val Leu Ser
1  5 10 15
Ala Thr Ser Arg Gly Val Ala Gly Ala Leu Arg Pro Leu Val Gln Ala
20 25 30
Thr Val Pro Ala
35

<210> SEQ ID NO 39
<211> LENGTH: 233
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 39
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gagacattgga ccctaatcatt aggttctttt cagtcctttt gtcaccttct gagacattatt
tgaggaatcctct tctgaatgttct ttagaattttg tgaagattt attgatgtatt
tgcaaacatt aatgtgaatt ttaagttgaa tattttcagg cattcactta
taaagacac ttgtaagcct tggagtgtctc aatcaaaaa aaataaaaaa aaa

<210> SEQ ID NO 40
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 40
Met Leu Ala Thr Arg Arg Leu Leu Gly Trp Ser Leu Pro Ala Arg Val
1 5 10 15
Ser Val Arg Phe
20

<210> SEQ ID NO 41
<211> LENGTH: 102
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 41
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60
gtggctgcca cccggcaaaa aaaaaaaaa aaaaaaaaaa aaaaaaaaaa a
102

<210> SEQ ID NO 42
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 42
Met Phe Phe Ser Ala Leu Arg Ala Arg Ala Ala Gly Leu Thr Ala
1 5 10 15
His Trp Gly Arg His Val Arg Asn Leu His Lys Thr Ala Met Gln Asn
20 25 30
Gly Ala Gly Gly Ala Leu Phe Val His Arg Asp
35 40

<210> SEQ ID NO 43
<211> LENGTH: 97
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 43
ttatatgtga actgttacaa tgccttaaaat gaataaaat atggacttcc aatctacgta
60
aaaaaaaaa aaaaaaaaa aaaaaaaaa aaaaaaaaa
97

<210> SEQ ID NO 44
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 44
Met Leu Arg Ala Ala Ala Arg Phe Gly Pro Arg Leu Gly Arg Arg Leu
1 5 10 15
Leu Ser Ala Ala Ala Thr Gln Ala
20
<210> SEQ ID NO: 45
<211> LENGTH: 450
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 45

gaatcatgca agttcttcct ctcagcattgt gatgaaagt tcagcaagat cagcaaaaaa 60
accaaaaaa atgatcctgg ggtgtcaaat attggaaaaag aagaaatattt cctacaaaat 120
cctttgggtc aagaagtttc tagaatgttg aatgataaace atgttggggtt ggtgaggtt 180
aagagttcag gaggacctt ttaacgcga acaataactgc tagcttcagc gatgtattttt 240
aaaaataga ttcacactgt ttctctctct ttgcaaaaggct ttctataaua tcgagtttat 300
agggaaaag aagctactatg tttacaatta tatcccccatt aagggactctg ttacacctg 360
cctggattttc tggcaaaaaa ttttttttta aaaaaaaaattagttgct cttaaaaaaa 420
aaaaaaaaaaaaaaaaaaaaaaaaaa 450

<210> SEQ ID NO: 46
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 46

Met Ala Ala Ser Pro His Thr Leu Ser Ser Arg Leu Leu Thr Gly Cye
1  5 10 15
Val Gly Gly Ser
20

<210> SEQ ID NO: 47
<211> LENGTH: 1424
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 47

gagcaagtggg aagccacacg cccctttccc ttcgctgcata gggagacatg ttggtgataa 60
tccggacacc aagaagagca attgctggggtt ttgaaacaga attataaacgc aatccggtgc 120
ccaggctgat ttcgaagctttttttt ttaataatta cccaaatgtg tcccacaaata 180
agaatgctat cagctcagtc aatgaataca aaaaaggaat ttttctttttc tttgagggtc 240
tttatatact cttccgccaa cccacacccc tttctgttcc ttctctctrc acatggggtt 300
acacatcaca agttctccct tttggctccaa tttctaccac cacaccccaac gcaacacca 360
catggccagc aagctgacag ttcgctgcata gaaagttgc gcctccagat ctcgctgtctg 420
tagttcatgt aagctcagttc cttcagaggc cttcgagcag cccctctctcg gttgaatgac 480
cagggctgtg aattttggtt ttccccaccc cacacatttc cttctotaaac 540
ataccatacg ctaggaccccg gtcgctgctg acgtgagcagc gggtttccac gatgtttgcct 600
tcgagagtc aagctgtccag gcagccccct gtctctctct caccacccctt ggttgtagac 660
atctcaagct cggacaggttt tttatagttg acctaatctg acagcatactg 720
ttggagacag tttcttcatg aaggggtcgc ctggaaacata caccagccag atctcttgcg 780
ccccagcccc attacagctt cctggagact actaatgtgg gcgcagctcct ctcacagtaa 840
cagcagcgt ttttcaaggc tgtatcgtga agggagttga ggaagaggg tgtgctgggc 900
| taaccagccc acagagctca cattctgtgc cctggtgta aaaaatcatg tcctcctctga | 960 |
| tatctcctga atctcagaat tagctccccc atgtgaatct ggttaaagag ccagaagcag |
| ggttttagga aattttaagg gtctctctctgt gtctctctgt ctctctctctctta | 1020 |
| ggttttagga aattttaagg gtctctctctgt gtctctctctgt ctctctctctctta |
| tattactctgtaggtgtagtgc cccggggtct gcagagccc cttgccccac | 1140 |
| aggtctttgaag atgtttttctatctcaggtc ccagggcac tgctgctgct | 1200 |
| tagagcgatac ctagctgccg tttgagaggtcttctcctcacttt | 1260 |
| aggtttttttgtagttcacc tccaactgt ccagggcac tgctgctgct | 1320 |
| tttttttttttttt tttttttttttttt tttttttttttttt tttttttttttttt | 1380 |
| aagactcctac aaccttcctgtgtagtgc ccagagcag cgagagctcc | 1424 |

<210> SEQ ID NO 48
<211> LENGTH: 760
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 48

Met Ala Pro Tyr Ser Leu Leu Val Thr Arg Leu Glu Lys Ala Leu Gly
1 5 10 15
Val Arg Glu Tyr His Val Ala Ser Val Leu Cys Gin Gin Arg Ala Lys Val
20 25 30
Ala Met Ser His Phe Glu Pro Asn Glu Tyr Ile His Tyr Asp Leu Leu
35 40 45
Glu Lys Asn Ile Asn Ile Val Arg Arg Leu Asn Arg Pro Leu Thr
50 55 60
Leu Ser Glu Lys Ile Val Tyr Gly His Leu Asp Asp Pro Ala Ser Gin
65 70 75 80
Glu Ile Glu Arg Gin Gin Ser Tyr Leu Arg Leu Arg Pro Gin Arg Gin
85 90 95
Ala Met Gin Gin Ser Ala Thr Ala Gin Met Ala Met Gin Gin Gin Thr Thr
100 105 110
Ser Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gins
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Lys Ser Phe Ala Arg Ile His Glu Thr Asn Leu Lys Lys Gln Gly Leu 690 695 700
Leu Pro Leu Thr Phe Ala Asp Pro Ala Asp Tyr Asn Lys Ile His Pro 705 710 715 720
Val Asp Lys Leu Thr Ile Gln Gly Leu Lys Asp Phe Thr Pro Gly Lys 725 730 735
Pro Leu Lys Cys Ile Ile Lys His Pro Asn Gly Thr Gln Glu Thr Ile 740 745 750
Leu Leu Asn His Thr Phe Asn Glu Thr Gln Ile Glu Trp Phe Arg Ala 755 760 765
Gly Ser Ala Leu Asn Arg Met Lys Glu Leu Gln Gln 770 775 780

<210> SEQ ID NO 49
<211> LENGTH: 782
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<405> SEQUENCE: 49

Met Leu Ser Arg Ala Val Cys Gly Thr Ser Arg Glu Leu Ala Pro Ala 1 5 10 15
Leu Gly Tyr Leu Gly Ser Arg Gln His Ser Leu Pro Asp Leu Pro 20 25 30
Tyr Asp Tyr Gly Ala Leu Glu Pro His Ile Asn Ala Gln Ile Met Gln 35 40 45
Leu His His Ser Lys His His Ala Tyr Val Asn Aen Leu Aen Val 50 55 60
Thr Glu Glu Lys Tyr Gln Glu Ala Leu Ala Lys Gly Asp Val Thr Ala 65 70 75 80
Gln Ile Ala Leu Gln Pro Ala Leu Lys Phe Asn Gly Gly Gly His Ile 95 100 90 95
Asn His Ser Ile Phe Trp Thr Asn Leu Ser Pro Asn Gly Gly Gly Glu 100 105 110
Pro Lys Gly Glu Leu Leu Glu Ala Ile Lys Arg Asp Phe Gly Ser Phe 115 120 125
Asp Lys Phe Lys Glu Leu Thr Ala Ala Ser Val Gly Val Gln Gly 130 135 140
Ser Gly Trp Gly Trp Leu Gly Phe Asn Lys Glu Arg Gly His Leu Gln 145 150 155 160
Ile Ala Ala Cys Pro Asn Gln Asp Pro Leu Gln Gly Thr Thr Gly Leu 165 170 175
Ile Pro Leu Leu Gly Ile Asp Val Trp Glu His Ala Tyr Tyr Leu Gln 180 185 190
Tyr Lys Asn Val Arg Pro Asp Tyr Leu Lys Ala Ile Trp Asn Val Ile 195 200 205
Asn Trp Glu Asn Val Thr Glu Arg Tyr Met Ala Cys Lys Lys 210 215 220

<210> SEQ ID NO 50
<211> LENGTH: 529
<212> TYPE: PRT
-continued

Leu Ser Arg Ala Ile Ala Glu Gly Ile Tyr Pro Ala Val Asp Pro
385 390 395 400
Leu Asp Ser Thr Ser Arg Ile Met Asp Pro Asn Ile Val Gly Ser Glu
405 410 415
His Tyr Asp Val Ala Arg Gly Val Gln Lys Ile Leu Gln Asp Tyr Lys
420 425 430
Ser Leu Gln Asp Ile Ile Ala Ile Leu Gly Met Asp Glu Leu Ser Glu
435 440 445
Glu Asp Lys Leu Thr Val Ser Arg Ala Arg Lys Ile Glu Arg Phe Leu
450 455 460
Ser Glu Pro Phe Glu Val Ala Glu Val Phe Thr Gly His Met Gly Lys
465 470 475 480
Leu Val Pro Leu Gly Thr Val Ile Gly Phe Glu Glu Ile Leu Ala
485 490 495
Gly Glu Tyr Asp His Leu Pro Glu Gln Ala Phe Tyr Met Val Gly Pro
500 505 510
Ile Glu Glu Ala Val Ala Lys Ala Asp Lys Leu Ala Glu Glu His Ser
515 520 525
Ser

<210> SEQ ID NO 51
<211> LENGTH: 274
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 51

Met Leu Ser Val Ala Ala Arg Ser Gly Pro Phe Ala Pro Val Leu Ser
1 5 10 15
Ala Thr Ser Arg Gly Val Ala Gly Ala Leu Arg Pro Leu Val Gin Ala
20 25 30
Thr Val Pro Ala Thr Pro Glu Gin Pro Val Leu Asp Leu Lys Arg Pro
35 40 45
Phe Leu Ser Arg Glu Ser Leu Ser Gly Gin Ala Val Arg Arg Pro Leu
50 55 60
Val Ala Ser Val Gly Leu Ann Val Pro Ala Ser Val Cys Tyr Ser His
65 70 75 80
Thr Asp Ile Lys Val Pro Asp Phe Ser Glu Tyr Arg Leu Glu Val
85 90 95
Leu Asp Ser Thr Lys Ser Ser Arg Glu Ser Ser Glu Ala Arg Lys Gly
100 105 110
Phe Ser Tyr Leu Val Thr Gly Val Thr Thr Val Gly Val Ala Tyr Ala
115 120 125
Ala Lys Asn Ala Val Thr Gin Phe Val Ser Ser Met Ser Ala Ser Ala
130 135 140
Asp Val Leu Ala Leu Ala Lys Ile Glu Ile Lys Leu Ser Asp Ile Pro
145 150 155 160
Glu Gly Lys Ann Met Ala Phe Trp Arg Gly Lys Pro Leu Phe Val
165 170 175
Arg His Arg Thr Gin Lys Glu Ile Glu Glu Gin Ala Val Glu Leu
180 185 190
Ser Gin Leu Arg Asp Pro Gin His Asp Leu Asp Arg Val Lys Lys Pro
195 200 205
Glu Trp Val Ile Leu Ile Gly Val Cys Thr His Leu Gly Cys Val Pro
210 215 220
Ile Ala Asn Ala Gly Asp Phe Gly Gly Tyr Tyr Cys Pro Cys His Gly
225 230 235 240
Ser His Tyr Asp Ala Ser Gly Arg Ile Arg Leu Gly Pro Ala Pro Leu
245 250 255
Asn Leu Glu Val Pro Thr Tyr Glu Phe Thr Ser Asp Asp Met Val Ile
260 265 270
Val Gly

<210> SEQ ID NO 52
<211> LENGTH: 464
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 52
Met Leu Ala Thr Arg Arg Leu Leu Gly Trp Ser Leu Pro Ala Arg Val
1  5  10  15
Ser Val Arg Phe Ser Gly Asp Thr Thr Ala Pro Lys Thr Ser Phe
20  25
Gly Ser Leu Lys Asp Glu Asp Arg Ile Phe Thr Asn Leu Tyr Gly Arg
35  40  45
His Asp Trp Arg Leu Lys Gly Ser Leu Ser Arg Gly Asp Trp Tyr Lys
50  55  60
Thr Lys Glu Ile Leu Leu Gly Pro Asp Trp Ile Leu Gly Glu Ile
65  70  75  80
Lys Thr Ser Gly Leu Arg Gly Arg Gly Gly Ala Gly Phe Pro Thr Gly
85  90  95
Leu Lys Trp Ser Phe Met Asn Lys Pro Ser Asp Gly Arg Pro Lys Tyr
100 105 110
Leu Val Val Asn Ala Asp Glu Gly Glu Pro Gly Thr Cys Lys Asp Arg
115 120 125
Glu Ile Leu Arg His Asp Pro His Leu Leu Gly Cys Leu Val
130 135 140
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145 150 155 160
Glu Phe Tyr Asn Glu Ala Ser Asn Leu Gin Val Ala Ile Arg Glu Ala
165 170 175
Tyr Glu Ala Gly Leu Ile Gly Asn Ala Cys Gly Ser Gly Tyr Asp
180 185 190
Phe Asp Val Phe Val Val Arg Gly Ala Gly Ala Tyr Ile Cys Gly Glu
195 200 205
Glu Thr Ala Leu Ile Glu Ser Ile Glu Gly Lys Gin Gly Lys Pro Arg
210 215 220
Leu Lys Pro Pro Phe Pro Ala Asp Val Gly Val Phe Gly Cys Pro Thr
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Pro Gly Phe Gly Val Gln Ala Gly Leu
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<211> LENGTH: 517
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<213> ORGANISM: Homo sapiens

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50 55 60
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65 70 75 80
Lys Ala Ala Arg Ala Phe Gln Leu Gly Ser Pro Trp Arg Arg Met
85 90 95
Asp Ala Ser His Arg Gly Arg Leu Leu Asn Arg Leu Ala Asp Leu Ile
100 105 110
Glu Arg Asp Arg Thr Tyr Leu Ala Leu Glu Thr Leu Asp Asn Gly
115 120 125
Lys Pro Tyr Val Ile Ser Tyr Leu Val Asp Leu Asp Met Val Leu Lys
130 135 140
Cys Leu Arg Tyr Ala Gly Trp Ala Asp Lys Tyr His Gly Lys Thr
145 150 155 160
Ile Pro Ile Asp Gly Asp Phe Phe Ser Tyr Thr Arg His Glu Pro Val
165 170 175
Gly Val Cys Gly Gln Ile Ile Pro Trp Asn Phe Pro Leu Leu Met Gln
180 185 190
Ala Trp Lys Leu Gly Pro Ala Leu Ala Thr Gly Asn Val Val Val Met
195 200 205
Lys Val Ala Glu Gln Thr Pro Leu Thr Ala Leu Tyr Val Ala Asn Leu
210 215 220
Ile Lys Glu Ala Gly Phe Pro Pro Gly Val Val Asn Ile Val Pro Gly
225 230 235 240
Phe Gly Pro Thr Ala Gly Ala Ile Ala Ser His Glu Asp Val Asp
245 250 255
Lys Val Ala Phe Thr Glu Ser Thr Glu Ile Gly Arg Val Ile Gln Val
260 265 270
Ala Ala Gly Ser Ser Asn Leu Lys Arg Val Thr Leu Glu Leu Gly Gly
275 280 285
Lys Ser Pro Asn Ile Ile Met Ser Asp Ala Asp Met Asp Trp Ala Val
The image contains a table of amino acid sequences from a scientific document. The sequences are presented in a linear format with each amino acid listed in order. The sequences appear to be part of a protein or peptide, possibly from a biological organism, indicated by the ORGANISM field which mentions Homo sapiens, a human biological organism. The sequences are numbered from 290 to 300, and the type of sequence data is indicated as PRT (Protein). The table continues from the previous page, likely providing more sequences of interest beyond what is shown here. The sequences are described in a plain text format, with each amino acid listed in a row, corresponding to their position in the sequence. The table structure remains consistent, with each row indicating the position and amino acid type.
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<213> ORGANISM: Homo sapiens  

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gggtcctga gatctactctg gacatcagc aggtatttg tagttggcga gtcttttttta 180  
tctagagtg agatctttta aagttgaagg gaaaatttaa ttttttaaa aacaccagtcg 240  
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gaaccccaag gttttagag ccaagagaca ggaatcaca ttgtggtcct ctgtgtctct 420  
gagtgggaca agggagcttt gattagctac tccggttcaca cacactgttaa gatacaaaac 480  
catctccaa tttgaagag agtagaaagtt tttctatagc agtaggtgct caaacaattc 540  
agcaacagtc aatcaggg aggggagaag gaatgaatgt gaaaggagaag ctaggacct 600  
tctctctac tactactact ttactagct actacttttt atagtaaacc ttcacagc 660  
acccagca tc tcagtttctt gcttcttttt atctgcactg tggtaagggg 720  
ctttttttaa aactaaagcag aatagcctaa gttataagct cccaaaggtt tocttttccc 780  
ttcacacttc tgtgctgtgg actacagtc acgactgtga tgtatttttt tcaagtgaatt 840  
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ataatgaaaa aaaaaaaaaa aa 922
<210> SEQ ID NO 58
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE: 
<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic Primer

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ggtgggttgg atctctgtgg ctcctggatat gggtggcgcg ggggagagtta ctct 240
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tctcgcggag cgtgtcaagg cctagctgac cgggtgcgtg agct 360
cggagcagca cccctgcaat ccagcctggt gaggagctgc cggcagagctg ctcttca 420
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1. An expression vector configured to deliver mRNA encoding a protein into the mitochondrion of a mammalian cell, said vector comprising:
   (i) a mitochondrion-targeting nucleic acid sequence (MTS);
   (ii) a nucleic acid sequence encoding said protein in accordance with a universal genetic code (CDS); and
   (iii) a 3'UTR nucleic acid sequence, located 3' of said CDS, wherein
   said MTS comprises a cDNA sequence of a MTS of a nuclear encoded mitochondrion-targeted mRNA,
   said 3'UTR nucleic acid sequence comprises a 3'UTR sequence of a nuclear-encoded mitochondrion-targeted
   mRNA,
   said vector does not comprise any sequence identical to a 3'UTR of a naturally occurring mRNA which is a
   nuclear-transcribed but not mitochondrion-targeted mRNA, a cDNA sequence of said 3'UTR of a naturally
   occurring mRNA, or a DNA sequence coding for said 3'UTR of a naturally-occurring mRNA in accordance
   with the universal genetic code, and
   said MTS and said 3' UTR nucleic acid sequence are configured to target mRNA expressed by said expression
   vector from a nuclear compartment to mitochondrion-bound polyosomes of a mammalian cell.

2. The expression vector of claim 1, wherein said MTS nucleic acid sequence comprises the MTS nucleic acid
   sequence of SOD2, COX10, ACO2, ATP5b, UQCRFS1, NDUFV1, NDUFV2, or ALDH2.

3. The expression vector of claim 1, wherein said MTS nucleic acid sequence comprises the MTS nucleic acid
   sequence of COX10.

4. The expression vector of claim 1, wherein said MTS nucleic acid sequence codes for a peptide selected from the
   group consisting of SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42,
   SEQ ID NO: 44, and SEQ ID NO: 46.

5. The expression vector of claim 1, wherein said MTS nucleic acid sequence codes for the peptide of SEQ ID NO:
   46.

6. The expression vector of claim 1, wherein said 3'UTR nucleic acid sequence comprises the 3'UTR sequence of
   SOD2, COX10, ACO2, ATP5b, UQCRFS1, NDUFV1, NDUFV2, ALDH2, or AK2.

7. The expression vector of claim 1, wherein said 3'UTR nucleic acid sequence comprises the 3'UTR sequence of
   COX10.

8. The expression vector of claim 1, wherein said 3'UTR nucleic acid sequence comprises the nucleic acid sequence
   selected from the group consisting of SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41,
   SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 57, and SEQ ID NO: 59.

9. The expression vector of claim 1, wherein said 3'UTR nucleic acid sequence comprises the nucleic acid sequence of
   SEQ ID NO: 59.

10. The expression vector of claim 1, wherein said MTS nucleic acid sequence comprises the MTS nucleic acid
    sequence of COX10, and said 3'UTR nucleic acid sequence comprises the 3' UTR nucleic acid sequence of COX10.

11. The expression vector of claim 1, wherein said nucleic acid sequence encoding said protein (CDS) encodes a
    naturally-occurring mitochondrial protein.

12. The expression vector of claim 11, wherein said nucleic acid sequence is recoded in accordance with the universal
    genetic code.

13. The expression vector of claim 11, wherein said naturally-occurring mitochondrial protein is selected from the
    group consisting of ATP6, ND1, and ND4.

14. The expression vector of claim 11, wherein said nucleic acid sequence encoding the naturally-occurring mitochondrial
    protein is selected from the group consisting of SEQ ID NO: 29, SEQ ID NO: 28, and SEQ ID NO: 27.

15. The expression vector of claim 1, wherein said mammalian cell is a human cell.