Transfection of mitochondria: strategy towards a gene therapy of mitochondrial DNA diseases

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ABSTRACT

Successes in classical gene therapies have been achieved by placing a corrected copy of a defective nuclear gene in cells. A similar gene replacement approach for a mutant mitochondrial genome is invariably linked to the use of a yet unavailable mitochondrial transfection vector. Here we show that DNA coupled covalently to a short mitochondrial leader peptide (chimera) can enter mitochondria via the protein import pathway, opening a new way for gene-, antisense-RNA- or antisense-DNA-delivery in molecular therapies. The import behavior of the purified chimera, composed of the amino-terminal leader peptide of the rat ornithine transcarbamylase (OTC) and a double stranded DNA molecule (17 bp or 322 bp), was tested by incubating with coupled and 'energized' rat liver mitochondria in the presence of reticulocyte lysate. The chimera was translocated with a high efficiency into the matrix of mitochondria utilizing the protein import pathway, independent from the size of its passenger DNA.

INTRODUCTION

Although eukaryotic cells are extremely diverse in structure and function, the metabolic pathway by which they satisfy their energy demand is conserved and relies strongly on mitochondrial oxidative phosphorylation (OXPHOS). Each cell contains therefore hundreds of mitochondria, enough to maintain a sufficient source of energy, so that organ integrity and function can be ensured. The main function of mitochondria, the synthesis of ATP by OXPHOS, is controlled by genes of the nuclear and the mitochondrial genome (mtDNA). Thus, thirteen essential subunits of the OXPHOS, including seven subunits of complex I (NADH:ubiquinone oxidoreductase), one subunit of complex III (ubiquinol:cytochrome c oxidoreductase), three subunits of complex IV (cytochrome c oxidase) and two subunits of complex V (ATP synthase), as well as the major part of the mitochondrial translation system (two rRNAs and 22 tRNAs), are encoded by the mitochondrial genome (1). Decreased OXPHOS capacities, as a result of homo- or heteroplasmic single base substitutions or large scale deletions of the mtDNA, have been recently associated with a broad spectrum of clinical manifestations, including

blindness, deafness, dementia, movement disorders, weakness, cardiac failure, diabetes, dystonia, renal dysfunction and liver disease (2–8). The severity of patient's symptoms caused by OXPHOS defects varies, depending on the nature of the mutation, the percentage of mutant mtDNAs in different tissues and the relative reliance of the affected organ systems on mitochondrial energy production (2). Thus, as mitochondrial ATP generation declines, it successively falls below the minimum energetic levels necessary for each organ to maintain a normal function (energetic threshold). This results in a progressive increase in the number and severity of clinical symptoms, with the visual system, the central nervous system, muscle, heart, pancreatic islets, kidney and liver being affected (2).

The molecular pathogenesis of these disorders is complex and heterogeneous: amino acid replacements in Leber's hereditary optic neuropathy [LHON; (9)] or in neuropathy, ataxia and retinitis pigmentosa [NARP; (10)] lead to functional defects in single OXPHOS complexes, whereas mutations in mitochondrial tRNA genes in patients presenting with myoclonic epilepsy and ragged-red fiber disease [MERRF; (11,12)], mitochondrial encephalomyopathy, lactic acidosis and stroke-like symptoms [MELAS; (13)], maternally transmitted diabetes mellitus [MTDM type II; (14)] and chronic progressive external ophthalmoplegia [CPEO; (15)] impair the overall mitochondrial translation or alter the transcription and thus limit the OXPHOS capacity (16,17), as it has been shown for mtDNA deletions in cases of CPEO (18) and adult-onset diabetes mellitus [AODM type II; (19)].

A wide variety of metabolic therapies have already been developed for treating patients with OXPHOS diseases (20). These involve supplementation with common OXPHOS cofactors and oxidizable substrates, stimulation of pyruvate dehydrogenase, and prevention of oxygen radical damage to mitochondrial membranes. While metabolic therapy holds the most promise for rapid development of effective regimes, such treatments are cumbersome, transient and thus not very reliable.

Somatic gene therapy has the potential to provide more permanent solutions to OXPHOS diseases. An essential factor when considering gene therapy approaches on mitochondrial DNA diseases is that classic gene therapy successes have been achieved by artificially placing a corrected copy of the abnormal gene into defective cells via viral or liposome mediated nucleic acid transfer. While a cytoplasmic/nuclear localization of the

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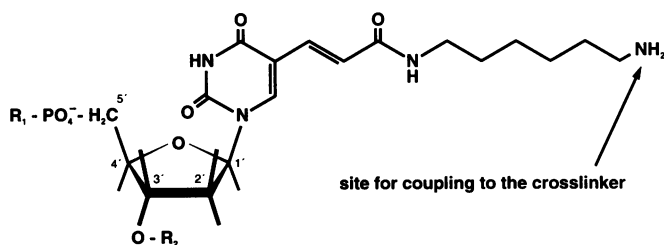* To whom correspondence should be addressed

Figure 1. Structure of the amino-modified deoxythymidine. The amino-modified dT (purchased as 5′-Dimethoxytrityl-5-[N-(trifluoroacetylamino-hexyl)-3-acrylimido]-2′-deoxyuridine, 3′-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite from Glen Research, Sterling, USA) is located within the center of the palindromic sequence of the synthesized oligonucleotide. The NH$_2$-group served as the site of conjugation to the heterobifunctional crosslinker. R1: nucleotides towards the 5′-end of the oligonucleotide; R2: nucleotides towards the 3′-end of the oligonucleotide.

corrected gene is sufficient to treat a nuclear gene defect, any such attempt on mitochondria is invariably linked to a yet unavailable targeting vector to the mitochondrial matrix.

To overcome this problem, we have developed a highly specific procedure to transfer nucleic acids into the matrix space of mitochondria that takes advantage of the existing protein import machinery and its capability to translocate signal peptide–DNA-conjugates (chimera) of different sizes into the matrix of mitochondria. The DNA moiety to be targeted ranged from a fragment of 17 bp (mimicking an antisense oligonucleotide) up to a fragment of as big as 322 bp (mimicking the smallest transcribable mitochondrial gene).

MATERIALS AND METHODS

Sequence and structure of the amino-modified oligodeoxynucleotide

A 39 nt oligodeoxynucleotide 5′-CCCCGGGTACCTTGCG AGCCCXGGGCTCGCAAGGTACCC-3′ was synthesized (Gene Assembler, Pharmacia) using an amino-modified deoxy-thymidine (X; amino-modified dT, Glen Research, Sterling, USA), located within the center of the palindromic sequence (see Fig. 1 for the chemical structure of the aminomodified dT). Thus, a stabilized secondary structure, composed of a loop, a stretch of 17 basepairs double stranded DNA and a sticky 5′-end, can be enforced by boiling (0.1 μM oligodeoxynucleotide) and subsequently shock-freezing of the sample in liquid nitrogen. After monomerization, an extra incubation at 65°C for 15 min did not alter the ratio of monomer to dimer, indicating the stability of the secondary structure of the monomer.

Coupling of peptide and DNA

The T4-polynucleotide kinase labelled oligonucleotide was incubated with a 20:1 molar excess of the bifunctional crosslinker maleimidobenzoyl-N-hydroxysuccinimide ester for 60 min at room temperature. Unreacted crosslinker was removed using a Nick-Spin-Column (Pharmacia), which had been equilibrated with PBS, followed by a 10 min incubation with 100 mM Tris–HCl, pH 7.0 and another gel filtration step. The recovered material was incubated with a 50-fold molar excess of the leader

peptide [the peptide-sequence derived either from the rat OTC (21–23) and was synthesized as a 33-mer H$_2$N-MLSNLRILLN-KAALRKAHTSMVRNFRYGKPVQC-COOH and a 43-mer H$_2$N-MLSNLRILLNKAALRKAHTSMVRNFRYGKPVQSQ VQLKPRDLC-COOH, or from the nuclear localization signal of the SV40 large T antigen (non-mitochondrial leader-sequence) and was synthesized as a 12-mer H$_2$N-CGYTPPKKKRKV-COOH (24,25); peptides were chemically synthesized, purified by HPLC and sequenced to verify their structures.] for 60 min at room temperature, followed by a 5 min incubation with 1 mM dithiothreitol to block excess unreacted maleimide groups. Usually 50% of the added DNA was converted to the peptide–DNA conjugate and was recovered from a preparative PAGE by electroelution.

Digestion of the chimera with proteinase K

100 ng of labelled chimera was incubated with 10 mM Tris–HCl (pH 7.8), 5 mM EDTA and 0.5% SDS in the presence of proteinase K (50 μg/ml) for 60 min at 37°C. The reaction products were loaded on a SDS–PAGE and visualized by autoradiography.

Labelling and attaching of a DNA fragment to the chimera

A mitochondrial DNA fragment was amplified with two primers (forward primer GGGGCCCTCTCCATACTACTAATCTCAT-CAATA, underlined nucleotides represent an artificial tail, necessary to create a sticky 5′-end; reverse primer: CTTGAT GCTTGTTCCTTTTGAT), annealing to nucleotides nt 469–490 and nt 762–741 respectively (1). The 5′-end of the reverse primer was radioactively labelled by T4 polynucleotide kinase and [γ-^{32}P]-ATP as a substrate prior to PCR amplification. Treatment of the amplified DNA with T4-DNA polymerase in the presence of 1 mM dGTP (3′–5′-exonuclease activity) resulted in a 5′-overhanging strand (26), compatible to the loop–stem structured oligonucleotide described above. Ligation product (DNA-extended chimera) was separated on an agarose gel and purified by electroelution.

Import assay conditions

Rat liver mitochondria were prepared from adult Wistar rats as described (27,28). ^{32}P-labelled chimera (1.4 × 10^6 c.p.m./pmol) were mixed with reticulocyte lysate (10% v/v) and import cocktail that contained 0.6 M sorbitol, 10 mM potassium phosphate (pH 7.4), 1 mM ATP, 2 mM MgCl$_2$ and 50 μM BSA (essentially fatty acid free). The mixture was incubated for 30 min at 25°C under gentle agitation. Mitochondria were re-isolated by centrifugation, resupended in import cocktail, supplemented with DNase I (2 U/μl), phosphodiesterase (PDE; 0.1 mU/μl) and calf intestinal phosphatase (CIP; 0.1 U/μl) and incubated for 10 min at 25°C under gentle agitation. After three washings, the resuspended mitochondria were layered on a sucrose gradient (1M–2M sucrose) and separated by centrifugation. Fractions were collected and characterized by measuring ^{32}P-radiation and cytochrome c-oxidase activity as described elsewhere (27,28). As control served an experiment carried out under identical conditions with equal amounts of the underivatized labelled oligo-deoxynucleotide (DNA, 1.4 × 10^6 c.p.m./pmol), in the absence and in the presence of an equal amount of signal peptide.

Disruption of mitochondria and mitoplasts

After import, mitochondria were treated with digitonin (1.2 µg for 10 µg mitochondrial protein) to remove the outer membrane and to release the intermembrane space. Mitoplasts were layered on a sucrose gradient (1M–2M) and separated by centrifugation. Fractions were collected and characterized by measuring marker enzymes for outer membrane/intermembrane space (adenylate kinase), inner membrane (cytochrome c-oxidase) and matrix space (malate dehydrogenase) as described (27,28). Fractions containing mitoplasts were pooled, disrupted by controlled lysis with lubrol (0.16 µg lubrol per 1 µg of mitoplasts) and subfractioned in matrix space and inner mitochondrial membranes by centrifugation (144.000 × g, 50 min, 5°C). Compartments were identified by measuring cytochrome c oxidase (inner membrane) and malate dehydrogenase (matrix) as described.

RESULTS

Synthesis and purification of the chimera

To study DNA uptake into mitochondria, a mitochondrial leader peptide was chemically synthesized and linked to different DNA species. The precursor peptide was composed of the 32 amino-terminal residues of the rat ornithine transcarbamylase (21–23), a mitochondrial matrix enzyme of the urea cycle, previously characterized to be imported into mitochondria without energy requirement on inner membrane passage (29). Its C-terminus was extended by an unique cysteine, necessary for the coupling of peptide to DNA. To allow a covalent linkage of DNA and peptide, an oligodeoxynucleotide (39 nt) was designed, harboring a reactive amino group at the center of its palindromic sequence (see Materials and Methods). Thus, a stable loop–stem-like secondary structure of the oligonucleotide can be enforced (see Fig. 2 and also 4), composed of the loop (containing the modified nucleotide), followed by 17 bp double-strand DNA moiety and four unpaired nucleotides at its 5'-end as a site for passenger DNA ligation. The '17 bp' oligo was linked to the leader peptide using a heterobifunctional crosslinker (see Figs 3 and 4). Approximately 50% of the DNA could be attached to the purified leader peptide. As expected, the adduct migrated more slowly on SDS–PAGE gels (Fig. 3, lane 1) and its protein moiety could be removed by proteinase K treatment (Fig. 3, lane 2).

Import into isolated mitochondria

To determine whether the mitochondrial protein import machinery recognizes the short signal peptide as a part of an artificial mitochondrial 'precursor DNA', we added the 5'-end labelled DNA–peptide conjugate to energized rat liver mitochondria. Mitochondria were re-isolated and resuspended in a 'shaving buffer'. The buffer contained DNase I, calf intestinal phosphatase (CIP) and phosphodiesterase (PDE) to determine whether the DNA moiety is protected by the membrane system. After import, mitochondria were fractioned by sucrose gradient centrifugation and analyzed for ^{32}P-radiation (chimera) and cytochrome c oxidase activity (marker enzyme for mitochondria). 57% of the chimera segregated with the fractions containing 91% of the overall cytochrome c-oxidase activity (mitochondria, fractions 5–7) and became inaccessible to further DNase I, CIP and PDE treatment, whereas 32% of the chimera segregated with the lightest fraction (fraction 1), harboring 2% of the overall

Figure 2. Monomerization of the aminomodified oligonucleotide. Native and monomerized oligonucleotides were separated on a 3% NuSieve^R + 1% SeaKem^R agarose gel and stained with ethidium bromide. Lanes 1 and 5: molecular weight marker (ΦX 174 RF DNA digested with HaeIII); lane 2: native oligodeoxynucleotide (monomer and dimer), lane 3: monomerized oligodeoxynucleotide (95°C, 5 min); lane 4: monomerized oligodeoxynucleotide (95°C, 5 min followed by incubation at 65°C, 15 min).
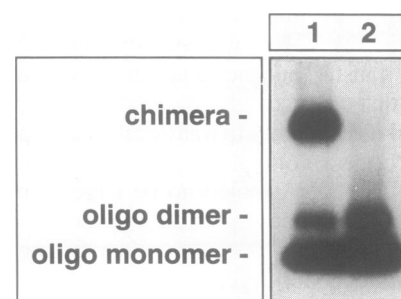

Figure 3. Protein moiety of the chimera is removed by proteinase K. The peptide–DNA conjugate was digested with proteinase K, the reaction products were analyzed on 17% SDS–PAGE and visualized by autoradiography. Lane 1: peptide–DNA conjugate (mitochondrial targeting signal, 43-mer); lane 2: peptide–DNA conjugate digested with proteinase K.

cytochrome c-oxidase activity (see Fig. 5A). Inaccessibility to the enzymes of the 'shaving buffer' was abolished when the mitochondrial membranes were solubilized with Triton X-100 (data not shown).

To exclude a possible unspecific DNA-uptake of mitochondria without a conjugated leader peptide, two experiments were carried out with equal amounts of underivatized 5'-labelled oligonucleotides in the presence and absence of the signal peptide under otherwise identical conditions. This time, less than 9% of the DNA was recovered with cytochrome c-oxidase activity (mitochondria, fractions 4–6), whereas 19% segregated with the lightest fraction (fraction 1), independent of the presence or absence of the signal peptide (see Fig. 5B). Thus, an unspecific DNA uptake mediated by the signal peptide could be largely excluded.

To proof, if the association of the chimeric molecule with the mitochondria is due to the mitochondria-specific leader peptide or simply depends on a hydrophobic interaction of the peptide moiety with membranes, an additional experiment was carried out with a chimeric molecule harboring a nuclear localization
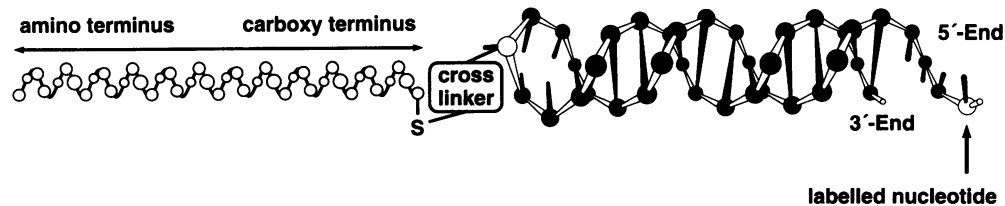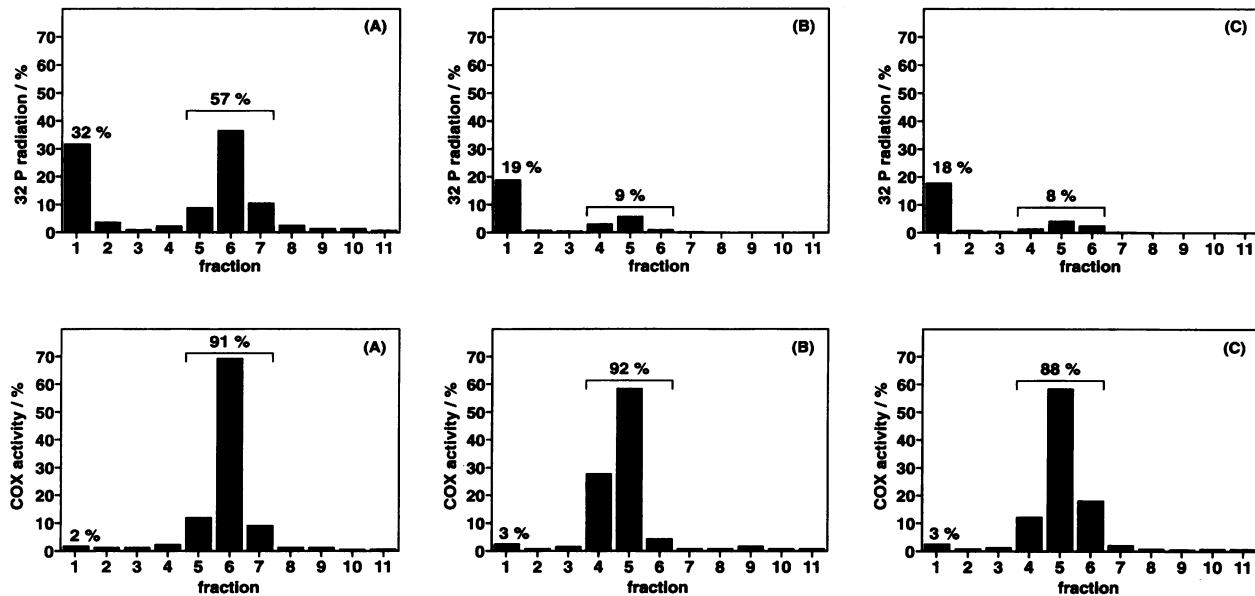
Figure 4. Model of the chimera.

Figure 5. Import of the chimera into isolated rat liver mitochondria. Radiation of the chimera [(A), upper left panel] is expressed as percent of the radiation loaded onto the gradient. Radiation of the DNA [(B), upper middle panel] and the DNA conjugated to the nuclear localization signal [(C), upper right panel] is expressed as percent radiation of the chimera, since equal amounts of chimera, DNA and DNA conjugated to the nuclear localization signal have been used for the import assay. Enzyme activity is displayed as percent activity loaded onto the gradient.

signal (24,25) as the protein moiety under otherwise identical conditions (see Materials and Methods). This time, less than 8 % of the chimera were recovered with the cytochrome c-oxidase activity (mitochondria, fractions 4–6, see Fig. 5C), whereas 18 % segregated with the lightest fraction (fraction 1, see Fig. 5C). Thus, an unspecific hydrophobic interaction of the chimera and the mitochondrial membrane system mediated by the protein moiety could be excluded.

To see whether the DNA uptake is limited only to small DNA molecules (< 17 bp), we attached a 301 bp dsDNA fragment via complementary 5'-ends to the chimera (DNA-extended chimera; see Fig. 6 and Materials and Methods). To ensure the complete removal of incomplete translocated molecules, only the free 5'-end of the attached passenger DNA was labelled. Thus, molecules which have been completely translocated through the membranes as well as partially translocated molecules protected with its 5'-end by the import channel are inaccesible to externally added PDE, DNAse I and CIP and contribute to the ^{32}P-radiation segregating with mitochondria, whereas DNA molecules sticking

in the channel with its 5'-labelled nucleotide outside the import pore as well as molecules, which are unspecifically bound to the outer mitochondrial surface, will be removed by externally added DNase I, PDE and CIP. Hence, they do not contribute to the ^{32}P-radiation segregating with the mitochondria. Import conditions were otherwise similar to these mentioned above. Results obtained from those experiments were in the same range as for the conjugated oligonucleotide itself, indicating that the DNA moiety is similarly protected by the mitochondrial membrane system. Thus, the amount of peptide–DNA-conjugate segregating on sucrose-gradients with mitochondria does not rely in the first place on the length of the attached passenger DNA.

Localizing the DNA-extended chimera within mitochondria

To test whether the DNA-extended chimera (passenger DNA attached to the peptide–DNA-conjugate) that was protected from externally added DNase I, PDE and CIP, has reached the soluble
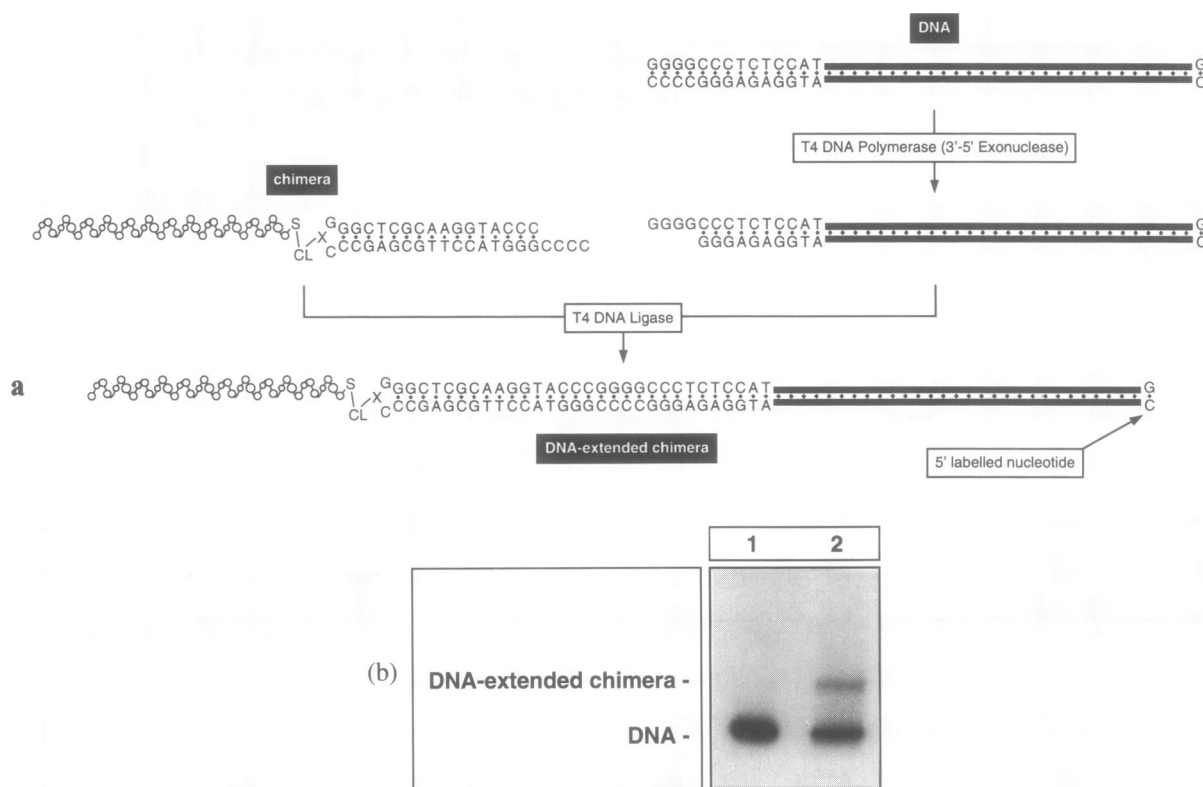
Figure 6. Attaching a 301 bp dsDNA fragment to the chimera (DNA-extended chimera). (a) Scheme of the synthesis. The treatment of the PCR amplified DNA with T4 DNA polymerase (3'–5' exonuclease) resulted in a single sticky 5'-end in the presence of dGTP. Ligation to the chimera resulted in a DNA-extended chimera (see Materials and Methods). Labelling of the DNA-extended chimera was carried out by labelling of the reverse primer using T4 polynucleotide kinase and [γ-^{32}P]-ATP prior to PCR amplification. (b) The ligation products were resolved on an agarose gel. Lane 1: PCR amplified dsDNA fragment of 301 bp; lane 2: ligation of the chimera to the amplified DNA; usually 40–50% of the added DNA was attached to the chimera.

matrix space, we subfractioned mitochondria into outer membrane/intermembrane space and inner membrane/matrix space by controlled lysis with the non-ionic detergent digitonin after import (see Materials and Methods). The compartments were identified by measuring compartment-specific enzyme activities (outer membrane/intermembrane space: adenylate kinase; inner membrane: cytochrome c oxidase; matrix space: malate dehydrogenase) and the uptake of the DNA-extended chimera (322 bp) was again followed by measuring the ^{32}P-radiation. As displayed in Figure 7A, 52% of the ^{32}P-radiation segregated with mitoplasts (8% adenylate kinase, 79% cytochrome c oxidase, 68% malate-dehydrogenase; fractions 4–8), whereas 42% of the radiation segregated with the lightest fraction (75% adenylate kinase, 4% cytochrome c oxidase, 31% malate-dehydrogenase; fraction 1). This is compatible with the distribution observed for the segregation of the chimera with mitochondria (57% with mitochondrial fractions, 52% with mitoplast fractions; compare Figs 5 and 7a), indicating that about 5% of the chimera could be removed by digitonin treatment and result in an increase of the lightest fraction of about 5%. However, the increase observed in the lightest fraction was about 10%, implying that other factors contribute as well. Depending on the specificity of the leader peptide, preproteins are directed during the import process to their compartments. Although a partially altered sorting of the molecules during the import assay into the intermembrane space

is unlikely, it cannot be excluded. In this context, mitoplasts released already 31% of their MDH activity, indicating that parts of the mitoplasts have been destroyed after import, either by the detergent digitonin, or by the centrifugal force during mitoplast separation. Hence, the extra increase of the lightest fraction is most likely due to peptide–DNA conjugates that had been already translocated into the mitochondrial matrix and were released during the partial disruption of the mitoplasts. The DNA-extended chimera, however, has been proven to undergo a similar density segregation on sucrose gradient as MDH (data not shown).

An inverse distribution was observed for the unconjugated DNA (underivatized aminomodified oligodeoxynucleotide ligated to the labelled passenger DNA; see Fig. 7B): 21% of the ^{32}P-radiation was detected within the lightest fraction (fraction 1), whereas less than 7% were segregating with fractions containing mitoplasts. Again, these data are fully compatible with the distribution observed for the segregation of unconjugated DNA with intact mitochondria (19% with the lightest fraction, 9% with the mitochondrial fractions, see Fig. 5B). Thus, the majority of the conjugated DNA (52%, excluding the portion released by the partial disruption of the mitoplasts) is associated with the inner membrane/matrix space of the mitochondria. To test, whether these molecules have been fully translocated into the mitochondrial matrix or stick to the inner mitochondrial mem-
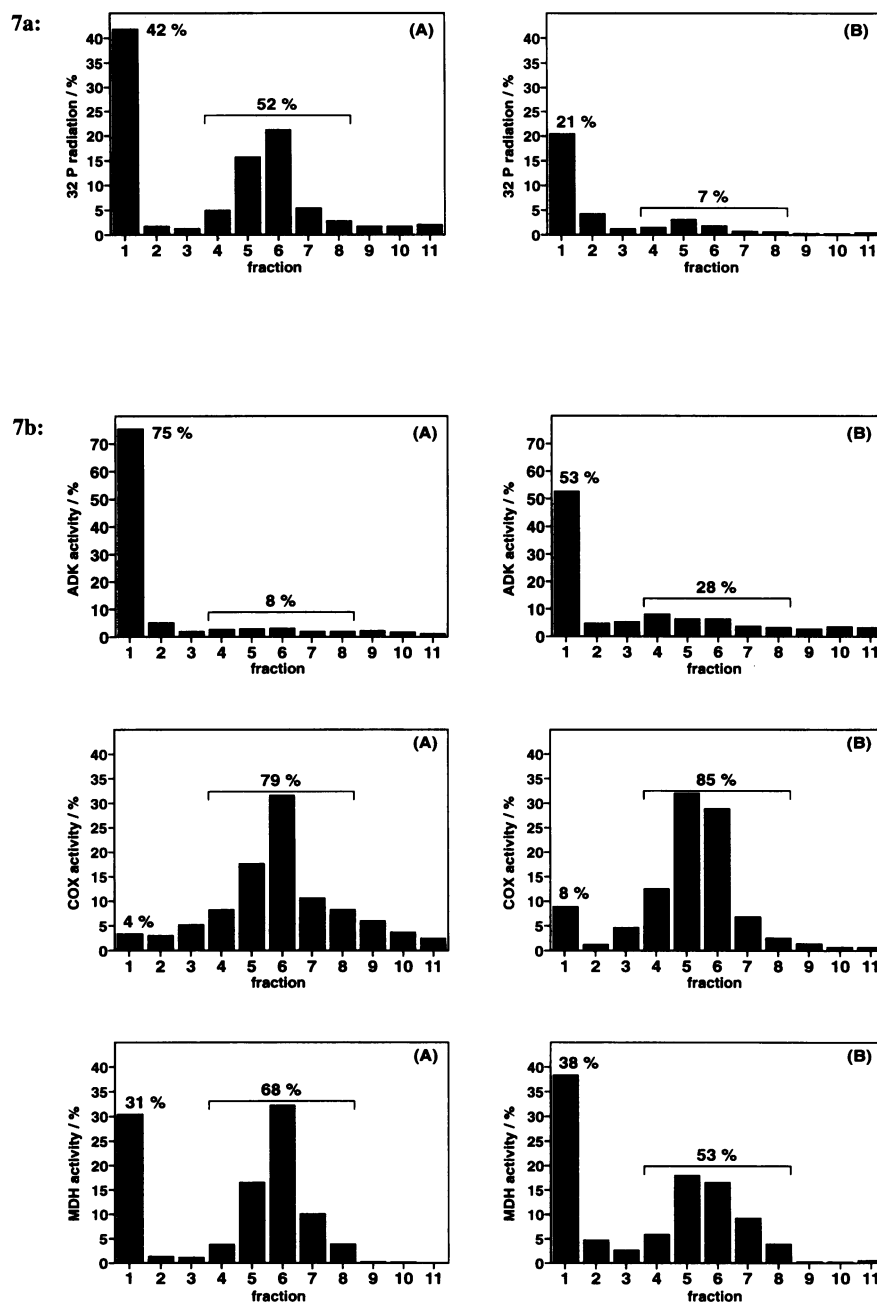
Figure 7. Localizing the DNA-extended chimera within mitochondrial compartments. After import, the mitochondrial outer membrane was stripped off by digitonin treatment. Mitoplasts were separated on a sucrose gradient (see Experimental Procedures). Displayed are the data obtained by a single experiment; repeated experiments led to similar results. (**7a**) The import of the DNA-extended chimera (**A**) and the DNA (**B**) was followed by measuring ^{32}P-radiation within the fractions. Radiation of the DNA-extended chimera is expressed as percent of the radiation loaded onto the gradient. Equal amounts of DNA-extended chimera and DNA have been used for the import assay, so that the radiation of the DNA is expressed as percent radiation of the chimera. (**7b**) Marker enzyme activities (ADK: adenylate kinase, intermembrane space; COX: cytochrome c oxidase, inner mitochondrial membrane; MDH: malate dehydrogenase, mitochondrial matrix) have been measured according to (27,28) and are displayed as percent activity loaded onto the gradient. (**A**): DNA-extended chimera; (**B**): DNA.

brane, the pooled fractions of mitoplasts (fractions 4–8, see Fig. 7) were further subfractioned into matrix space and inner mitochondrial membrane by lubrol treatment. Among the 52% of the conjugated DNA segregating with mitoplasts, 19% were released as part of the matrix (6% cytochrome c-oxidase, 91% malate-dehdrogenase), whereas 33% were recovered with the

membranes (94% cytochrome c-oxidase, 9% malate-dehydrogenase). Thus, 37% (19%) of the conjugated molecules segregating with mitoplasts have been completely translocated into the matrix, whereas 63% (33%) stuck to the inner mitochondrial membrane or have been entrapped within vesicles generated during mitoplast lysis. Due to the fact that the diameter of double

stranded DNA is about five times as big as of a fully extended linear polypeptide chain, the latter 63% could be explained by an arrest during import or by a delayed translocation process caused by the tight fitting of DNA and import pore.

Compatible with a slow uptake rate of the artificial molecules is the observation that mitochondria harboring a disrupted protein processing system are defective for translocation of radiolabelled precursor proteins (30). Thus, an unprocessible signal peptide could account for the observed incomplete or delayed transloca- tion. The import of precursor proteins involves specific binding to receptor molecules present in the outer mitochondrial mem- brane (31), translocation through one or both mitochondrial membranes by a process requiring energy at the step of inner membrane passage, and proteolytic cleavage of the NH_2-terminal leader sequence releasing the mature protein (32–35). Human ornithine transcarbamylase is known to be synthesized as a preprotein, cleaved twice after import (36). The two-step processing requires two distinct matrix proteases, mitochondrial processing peptidase (MPP) and mitochondrial intermediate peptidase (MIP). The mature OTC is formed by MIP that removes eight amino acids from the amino terminus of the intermediate (36–38), generated by MPP. Since a processible presequence could promote the import behaviour of the chimerical molecules, a pre-sequence of the rat OTC was synthesized with an extension at its C-terminus of 10 aminoacids of the mature protein (21,23) to ensure a cleavable signal peptide. The same experiments were carried out under otherwise identical conditions and resulted in a 30% increased release of molecules during lysis of mitoplasts compared to the experiment with the short leader peptide. The efficiency of the import seems to depend on the selected leader peptide and its processability.

DISCUSSION

In this study we have found that artificial molecules composed of a mitochondrial leader peptide and a DNA moiety can be imported into isolated mitochondria via the protein import pathway. The behaviour of the chimera, however, is not unexpected, because Vestweber and Schatz have been able to demonstrate with their experiments that mitochondrial ribonuclo- proteins required for mitochondrial RNA processing (mtRNP) could utilize the same pathway (39). In expanding their experiments, our data strongly suggest that a signal peptide is sufficient to allow a complete translocation of double-stranded DNA into the predicted compartment and that the transport is not limited to short single or double stranded oligonucleotides. The transport of DNA into the mitochondrial matrix increases, when the signal peptide comprises a part of the mature protein, ensuring that the mitochondrial protein processing system can act properly. These data are compatible with the observation that mitochondria harboring a disrupted protein processing system are impaired for translocation of radiolabelled precursor proteins (30), under- lining that the preprotein processing is an important step during protein and chimera import.

The unique loop–stem structure of the selected oligonucleotide provides two major advantages: first, it prevents a dissociation of complementary DNA strands during import, which cannot be ensured by using a linkage site at the oligos 5′-end. Second, its single-stranded 5′-end enabled us to attach a 301 bp dsDNA as a passenger to otherwise absolutely identical conjugated molecules so that a DNA moiety of 322 bp is generated. Thus, the import

behaviour of molecules differing in length are directly compar- able and an altered yield of imported molecules depends exclusively on the molecule's length. Our data show that the developed transfection strategy is not limited to small oligonu- cleotides. So far, to a size of 322 bp of the DNA moiety, the uptake efficiency was not altered in our hands. Again, this result is not completely unexpected, because the main rate limiting step for a functional translocation is incontestably linked to the diameter of the molecule to be imported. Vestweber and Schatz have been able to show with their experiments that artificial mitochondrial precursor proteins harboring internal disulfide bridges block the import of authentic precursors into mitochondria (40), while precursors containing branched polypeptides or highly charged organic molecules can be translocated into the matrix of the organelle (41). Hence, most likely the loop structure of the DNA (chimera) determines the uptake rate, whereas length and charge of the nucleic acids plays a secondary role, implying that this technique provides an extremely powerful and versatile tool to transfect mitochondria with nucleic acids of different lengths.

An important point when considering this method as a tool for a gene therapy approach on mitochondrial DNA diseases is that the classic gene therapy successes have been achieved by artificially placing a corrected copy of the abnormal gene into defective cells. Whilst penetrating the cell membrane via viral delivery systems is useless for the chimera in this context, an alternative approach may be possible by utilizing liposomes as a cellular transfection tool. It has been shown by Felgner *et al.* that synthetic cationic lipids can form small unilamellar liposomes capable of interacting spontaneously with DNA to form lipid– DNA complexes with 100% entrapment of the DNA (42,43). Furthermore, fusion of these complexes with the plasma mem- brane of tissue culture cells lead to both uptake and expression of the DNA. Once transfer of the chimera into the cytoplasm of the cell could be mediated by this technique, then any artificially nucleic acid could be taken up by mitochondria via the protein import pathway. It has to be stressed in this context that a DNA fragment of 300 bp in length is sufficient to allow the construction of a transcribable mitochondrial tRNA gene, composed of its coding sequence flanked by the nucleotides essential for RNA processing (about 150 nt) and a mitochondrial promotor (O_L, about 150 nt). Thus, single base replacements in tRNA genes that have been recently associated with MERRF, MELAS and maternally transmitted diabetes mellitus, could be treated with those constructs. Alternatively, a gene therapy approach could be based on the observation that mutant mitochondrial genomes have a replicative advantage (44,45). Considering the unique way of mtDNA replication, 'antisense'-DNA-oligonucleotides could be designed having a high affinity to the mutated mitochondrial genome during the single stranded replication phase, so that these oligos will selectively inhibit subsequent duplication of the mutated genomes, while wild-type genomes will be unaffected. After a sufficient period of time, the level of wild-type mtDNA relative to the mutated genomes could be promoted. This approach is of outmost interest for diseases associated with heteroplasmic mitochondrial DNA deletions, as adult onset diabetes mellitus, CPEO or Kearns–Sayre syndrome, since antisense oligodeoxynucleotides can be designed highly specific to the fusion breakpoint of the deleted mitochondrial genome. Hence, eliminating the replicative advantage of the mutated DNA could induce a segregation of heteroplasmic mtDNA populations towards homoplasmic wild-type mtDNA populations. Both

strategies, however, will most likely not lead to a 100% correction of all defective mitochondria. This is in turn not required, because it has been demonstrated that clinical symptoms become only manifest when the OXPHOS fidelity falls below a tissue specific energetic threshold (2). Thus, overcoming the energetic barrier either by importing a corrected copy of the defective gene or by eliminating the replicative advantage of the mutated genome might be sufficient to switch phenotype expression.

Protein-mediated transport of nucleic acids has been reported recently for adenovirus coupled to DNA–polylysine complexes and gains currently much attention for gene therapy approaches (46). Our strategy to use organellar and compartment specific signal peptides as a recognition sequence takes advantage of an evolutionary conserved and highly efficient protein targeting pathway. Hence, by selecting other signal sequences, this method will allow a specific targeting to other organelles as well (e.g. nucleus, chloroplast). Approaches on this basis may gain an extraordinary importance for the development of vectors that can be retained and expressed in nondividing cells.

ACKNOWLEDGEMENTS

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