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RESEARCH PAPER

DNA Delivery to Mitochondria: Sequence Specificity and Energy Enhancement

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ABSTRACT

Purpose Mitochondria are competent for DNA uptake *in vitro*, a mechanism which may support delivery of therapeutic DNA to complement organelle DNA mutations. We document here key aspects of the DNA import process, so as to further lay the ground for mitochondrial transfection in intact cells.

Methods We developed DNA import assays with isolated mitochondria from different organisms, using DNA substrates of various sequences and sizes. Further import experiments investigated the possible role of ATP and protein phosphorylation in the uptake process. The fate of adenine nucleotides and the formation of phosphorylated proteins were analyzed.

Results We demonstrate that the efficiency of mitochondrial uptake depends on the sequence of the DNA to be translocated. The process becomes sequence-selective for large DNA substrates. Assays run with a natural mitochondrial plasmid identified sequence elements which promote organellar uptake. ATP enhances DNA import and allows tight integration of the exogenous DNA into mitochondrial nucleoids. ATP hydrolysis has to occur during the DNA uptake process and might trigger phosphorylation of co-factors.

Conclusions Our data contribute critical information to optimize DNA delivery into mitochondria and open the prospect of targeting whole mitochondrial genomes or complex constructs into mammalian organelles *in vitro* and *in vivo*.

KEY WORDS DNA import · mitochondrial disease · mitochondrial plasmid · organelle transfection · protein phosphorylation

ABBREVIATIONS

bp	base-pair
BSA	bovine serum albumin
CCCP	carbonyl cyanide m-chlorophenylhydrazone
DEAE-cellulose	diethylaminoethyl-cellulose
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
kb	kilobase-pair
mtDNA	mitochondrial DNA
OXPHOS	oxidative phosphorylation
PCR	polymerase chain reaction
PEI-cellulose	polyethylenimine-cellulose
PMSF	phenylmethylsulfonyl fluoride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TLC	thin-layer chromatography

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INTRODUCTION

The human mitochondrial genome encodes 13 proteins and all the tRNAs and rRNAs required for their intra-mitochondrial synthesis. All of these polypeptides are components of large multi-subunit complexes found in the mitochondrial inner membrane. These complexes function to couple cellular respiration to the production of ATP, a process referred to as oxidative phosphorylation (OXPHOS). The identification of pathologies caused by mutations in the mitochondrial genome has been constantly increasing during the last decade (1). Clinical symptoms can be extremely variable and range from single-affected tissues to multisystemic syndromes. In general, high energy-demanding tissues, like brain, heart and muscle, are affected, which is consistent with defects in OXPHOS and makes mutations in the human mitochondrial DNA (mtDNA) a common cause of hereditary neuromuscular diseases. Both deletions and point mutations can affect mtDNA. Deletion size varies from a single base to several kilobases. Large deletions are associated with chronic progressive external ophthalmoplegia (CPEO), Kearns-Sayre syndrome (KSS) and Pearson's marrow-pancreas syndrome. Over a hundred point mutations have been characterized, causing a wide range of phenotypes, such as MELAS (myopathy encephalopathy with lactic acidosis), MERRF (myoclonic epilepsy and ragged red fibers), NARP (neuropathy ataxia and retinis pigmentosa), MILS (maternally inherited Leigh syndrom) and LHON (Leber heredity optic neuropathy). The nuclear background of a mtDNA mutation affects its biochemical expression, and the same mutation can be present in patients with different mitochondrial diseases. Mitochondrial DNA mutations can be homoplasmic or heteroplasmic. In the homoplasmic state, all mtDNA copies in the cells are mutated. Heteroplasmy refers to the coexistence of wild-type and mutant mtDNA populations. An important concept in mitochondrial diseases is the threshold effect, *i.e.* the percentage of mutant copies usually determines symptom expression. These degenerative disorders are currently incurable and would benefit from the development of gene replacement strategies. However, only yeast (*Saccharomyces cerevisiae*, *Candida glabrata*) and the unicellular alga *Chlamydomonas reinhardtii* are currently amenable to mitochondrial genetic transformation in intact cells (2,3). Conventional methodologies have failed so far to achieve transformation of mitochondria in plants or in mammalian cells. Alternative organelle transfection approaches and complementation strategies have been explored. Although patient treatment remains far ahead, promising data have been generated (4). In this respect, we demonstrated that isolated mitochondria are actually able to import linear DNA. Originally established for plant organelles (5), this new process was subsequently extended to mammalian (6) and *Saccharomyces cerevisiae* (7) mitochondria.

The discovery of mitochondrial competence for DNA uptake represents a further basis for transformation strategies, as the internalized substrates are fully functional in the organelles. DNA imported into isolated plant or mammalian mitochondria is transcribed *in organello* when carrying an appropriate mitochondrial promoter and the resulting transcripts are processed (5,6,8). Furthermore, DNA carrying specific lesions is repaired upon uptake into plant or mammalian organelles (9,10). Finally, an import substrate composed of a marker gene flanked by sequences homologous to mtDNA regions recombines with the resident DNA in plant mitochondria, leading to the integration of the marker gene (11). The way to organelle transformation in whole cells, however, is likely to be improved by a better understanding of the uptake process, which remains mostly unknown. Whereas the data imply an involvement of the voltage-dependent anion channel (VDAC) in DNA transport through the outer membrane (5–7), translocation through the mitochondrial inner membrane remains unclear. Optimizing DNA import and integration into isolated organelles is of particular relevance to the goal of transfecting mitochondria in intact cells as, at least in some cases, it seems possible to directly introduce isolated mitochondria carrying exogenous DNA into mammalian cells (12). We deepen here the investigations on the mechanism underlying DNA uptake into mitochondria, with special reference to substrate size and sequence, energy requirement, as well as possible dependence on phosphorylation of putative protein cofactors. Plant, mammalian and yeast organelles are considered.

MATERIALS AND METHODS

DNA Substrates for Mitochondrial Import Assays

The following substrates were used to analyze the size dependence of mitochondrial import efficiency. The 1.0 kb fragment was obtained upon PCR amplification of the *Carnation Italian ringspot virus* (CIRV) *orf1* cDNA (13) (accession No. X85215), using the previously constructed p36K plasmid (14) as a template and the two oligodeoxyribonucleotides CIRVd1 and CIRVc18 (see sequences below) as primers. The 2.3 kb DNA was the linear plasmid (accession No. X13704) from maize (*Zea mays*) mitochondria (15). It was amplified by PCR from maize mitochondrial DNA using the single primer NI2.3 kb, which annealed to the terminal inverted repeats of the 2.3 kb plasmid. The 4.9 and 6.4 kb linear DNA fragments were obtained upon *Bam*HI digestion of the previously constructed p36K and p95K plasmids (14). Finally, the 12.4 kb DNA corre-

sponded to the pBINPLUS expression vector (16) digested with *Hind*III. For substrates obtained by restriction enzyme digestion, sticky ends were filled with the Klenow fragment of *E. coli* DNA polymerase. After purification of the DNA on silica membrane columns (Nucleospin Extract II kit, Macherey-Nagel), 5′-phosphates were removed using alkaline phosphatase. The DNA was then purified by phenol/chloroform and chloroform extractions and ethanol-precipitated. For substrates obtained by PCR amplification, the DNA was purified with the Nucleospin Extract II kit (Macherey-Nagel). The five DNA substrates were finally end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Substrates were adjusted to a similar specific activity, and 20 fmol of each was used for import assays.

For further import experiments, the maize 2.3 kb linear plasmid was PCR-amplified with or without its terminal inverted repeats using the single primer NI2.3 kb or the pair of primers FL2.3int1/FL2.3int2, whereas the pBINPLUS plasmid was prepared as a PCR product generated with primers NIpBina and NIpBinb. Further assays also involved the 11.6 kb linear plasmid (accession No. AB073400) present in rapeseed (*Brassica napus*) and turnip (*Brassica rapa*) mitochondria (17), which was amplified by PCR from turnip mtDNA. It was generated with or without its terminal inverted repeats using either the single primer NIBnapIRpl or the couple of primers NI10.9A/NI10.9B. Finally, the entire 11.6 kb plasmid was cloned into the *Kpn*I restriction site of the pBluescript vector (Stratagene). The single *Eco*RI site of the vector was eliminated by deleting the *Sal*I-*Sma*I region in the multiple cloning site. The resulting plasmid was digested with *Eco*RI, and the restriction fragments were separated on an agarose gel. The fragment of 4.5 kb containing the pBluescript with nucleotides 1–467 and 10563–11640 of the *B. rapa* 11.6 kb plasmid was recovered, ligated so as to join nucleotide 467 to nucleotide 10563 and recloned. The remaining sequences of the 11.6 plasmid were subsequently PCR-amplified as above with or without the terminal inverted repeats using either the single primer NIBnapIRpl or the couple of primers NI10.9A/NI10.9B. The different DNA substrates were radiolabeled through a single PCR cycle in the presence of [α-^{32}P]dCTP as described previously (5,6). Long DNA substrates, around 10 kb or above, were amplified with the Expand Long Template PCR System (Roche) or the Long PCR Enzyme Mix (Fermentas).

Primer Sequences

CIRVd1: 5′-AAGATCCATGGAGGGTTTGAAGGCT-3′
CIRVc18: 5′-CACTAGAATTCTAAAACACAGACA GTTTTTCCCTG-3′
NI2.3 kb: AAAAGTATAGCAACACACAATAC
FL2.3int1: AACACACTACCTTATTGAGCATATT
FL2.3int2: GAGACTGTTCTAATCTTTTATTTATT
NIpBina: CTTGCATGCCTGAGTCTA
NIpBinb: CTTGGCGCGCCAGCTTGG
NIBnapIRpl: AAAAATACTACAAACTA TAGCTTCA
NI10.9A: TGAATTTGATGATTCAGAATTGTTC
NI10.9B: ATCAGACGAAAATTCAGATTCAGA

Isolation of Mitochondria

Plant mitochondria were extracted from potato (*Solanum tuberosum*) tubers or turnip (*Brassica rapa*) roots following previously described protocols (5,18). Isolation of potato mitochondria was on continuous Percoll gradients, whereas turnip organelles were purified on discontinuous gradients (18.5%, 21% and 45% Percoll, v/v). Potato mitoplasts were generated by hypotonic swelling of mitochondria and purified on sucrose gradients according to Heins *et al.* (19).

Yeast (*Saccharomyces cerevisiae*) mitochondria were prepared as before (7) from the parental BY4742 strain (*MAT*α, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0*) obtained from Euroscarf (Frankfurt, Germany). For preparation of yeast mitochondria in the presence of phosphatase inhibitors, 1/100 volume of Phosphatase Inhibitor Cocktails 1 and 2 (Sigma-Aldrich) was included in the buffers from spheroplast lysis to the end of the isolation. For assays involving incubation with ATP and analysis by SDS-PAGE, yeast mitochondria were further purified on a three-step sucrose gradient as described by Meisinger *et al.* (20).

For the isolation of human mitochondria, HepG2 hepatoma cells were grown to ~80% confluence, stripped from the flasks, diluted with cold PBS (sodium phosphate 10 mM, NaCl 150 mM, pH 7.2) and centrifuged at 500*g* for 5 min. All subsequent steps were performed at 4°C. The pellet was resuspended in extraction buffer (250 mM sucrose, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PMSF) before homogenization by 15 strokes in a hand-held glass/Teflon potter. The homogenate was centrifuged at 400*g* for 4 min. The supernatant was stored on ice, while the pellet was resuspended in extraction buffer, extracted again with the homogenizer and centrifuged at 400*g* for 4 min. The first and second supernatants were pooled and centrifuged at 15,000*g* for 10 min. The further pellet was resuspended in a small volume of extraction buffer with a loose potter, loaded on top of a two-layer gradient (1/4 part of 60% w/v sucrose in 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1% w/v BSA and 3/4 parts of 50% v/v Percoll in extraction buffer) and centrifuged for 40 min at 100,000*g*. Intact mitochondria were recovered from the interface between the sucrose and Percoll layers, diluted 10 times with extraction buffer and centrifuged for 10 min at 18,000*g*. A second wash was performed in extraction buffer without BSA, and mitochondria were resuspended in

import buffer prior to estimating protein concentration. Mitochondrial preparations were routinely checked for their integrity and respiratory control (5).

Import Assays

Standard DNA import assays with plant, human and yeast mitochondria were carried out according to previously described methodologies (5–7). When running assays with human cell mitochondria, the final nucleic acid pellets were resuspended in 120 µl of 10 mM Tris-HCl pH 7.5, 1 mM EDTA containing 200 µg of proteinase K, and incubated for 2 h at 55°C followed by 16 h at 37°C. After addition of 200 µg of proteinase K, the samples were further incubated at 55°C for 2 h. Nucleic acids were finally re-purified by phenol/chloroform extraction and ethanol precipitation before standard analysis.

For incubation with [α-^{32}P]ATP or [α-^{32}P]AMP, yeast mitochondria corresponding to 300 µg of proteins were incubated at 30°C during different times in 400 µl of 0.6 M mannitol, 40 mM Tris-HCl pH 7.25 buffer containing 2 mM $MgCl_2$, 2 mM ATP and 0.4 µl of [α-^{32}P]ATP (3,000 Ci/mmol, 1 mmol/l). After incubation, EDTA was added up to 10 mM, the samples were loaded onto a sucrose cushion (27% sucrose w/v, 10 mM Hepes-KOH pH 7.4) and centrifuged for 8 min at 18,000*g*. For each sample, a volume of 50 µl was recovered from the upper layer above the cushion and taken as the extra-mitochondrial fraction. The sucrose cushion was subsequently eliminated and the mitochondrial pellets were lysed in 6 µl of 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% v/v Triton X-100. After centrifugation for 10 min at 18,000*g*, the supernatants were recovered and taken as the intra-mitochondrial fractions. One microliter of each sample was spotted onto a PEI-cellulose or DEAE-cellulose TLC plate. Migration was developed in 1 M formic acid, 0.5 M LiCl (21) for PEI-cellulose plates or 7 M isobutyric acid, 0.13 M ammonia for DEAE-cellulose plates. Radioactivity was revealed by autoradiography. Unlabeled AMP, ADP and ATP markers were spotted on the same plate and visualized under UV light.

Phosphoprotein Detection

For protein phosphorylation analysis, purified yeast mitochondria corresponding to 150 µg of proteins were incubated at 30°C during 30 min in 200 µl of import buffer (0.6 M mannitol, 40 mM Tris-HCl pH 7.25) containing 2 mM $MgCl_2$ and 2 mM ATP. For assays with labeled ATP, 10 µl of [γ-^{32}P]ATP (3,000 Ci/mmol, 1 mmol/l) was added. After incubation, mitochondria were washed twice with 0.6 M mannitol, 40 mM Tris-HCl pH 7.25, 10 mM EDTA, 10 mM EGTA, and membrane proteins were extracted. For this, the organelle pellets resulting from the second washing step were freeze-thawed three times, resuspended in 200 µl potassium phosphate pH 7.2, 0.5 mM PMSF, sonicated three times for 15 s at 4°C and centrifuged at 100,000*g* for 15 min. The final pellets were resuspended in 45 µl of 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% v/v Triton X-100, and proteins were fractionated by SDS-PAGE on 13% w/v polyacrylamide gels. For samples from assays run in the presence of [γ-^{32}P]ATP, the general protein pattern was visualized by Coomassie Blue staining, and labeled phosphorylated proteins were subsequently detected by autoradiography. In the case of non-radioactive assays, phosphorylated proteins were detected by fluorescence using the Pro-Q Diamond phosphoprotein gel stain (Molecular Probes). Fluorescent staining was performed by fixing the gels in 45% v/v methanol, 5% v/v acetic acid overnight, washing three times for 10 min with water and incubating for 2 h in Pro-Q Diamond solution. Gels were finally washed three times for 30 min in 20% v/v acetonitrile, 50 mM sodium acetate pH 4.0 and two times in water. Fluorescence images were acquired on an Ettan DIGE Imager scanner (GE Healthcare) with a 540 nm excitation and 595 nm band pass emission filter. The general protein pattern was subsequently visualized by Coomassie Blue staining.

RESULTS

Size-Dependent Decrease of DNA Uptake Efficiency is Sequence-Dependent

We have shown that double-stranded, linear DNA can be imported into isolated mitochondria extracted from plants, rat liver, human cells and the yeast *Saccharomyces cerevisiae* (5–7,9,10). Various constructs have been successfully used in these studies, aiming to establish *in organello* transcription, repair or recombination of the imported DNA. Experiments with rat mitochondria indicated a decrease in the proportion of imported DNA when increasing the size of the substrate from 0.7 kb to 3.6 kb (6). However, the question of the maximal size that the process can accommodate has not been addressed so far. This is an important issue for further *in organello* studies and for the development of a putative organelle transformation strategy in whole cells.

To analyse the size limitations of mitochondrial import, double-stranded DNA fragments with various sequences ranging from 1.0 to 12.4 kb were 5′ end-labelled to a similar specific activity and used for import into isolated *Solanum tuberosum* (potato) mitochondria. The import assays were run with the same number of DNA molecules in each case. The results showed a similar uptake efficiency for

DNA fragments of 1 kb and 2.3 kb, but import efficiency of a 4.9 kb substrate was much lower (Fig. 1, lanes 1 to 3). Remarkably, whereas further increasing the size might be expected to further impair organellar uptake, the 6.4 kb fragment used in these experiments was actually more efficiently imported than the three smaller substrates (Fig. 1, lane 4). Conversely, in line with the original view of a size-limited process, uptake of the largest DNA substrate tested, *i.e.* the linearized 12.4 kb pBINPLUS expression vector, was not detectable (Fig. 1, lane 5). These results nevertheless implied that the sequence of the DNA fragment can positively influence mitochondrial import.

Import of Large (>7 Kb) DNA Substrates is Sequence-Selective

Further surprising observations were made with a particular plant mitochondrial genetic component. In addition to the main organelle genome, mitochondria of *Brassica napus* (rapeseed) and of some *Brassica rapa* (turnip) varieties contain an 11.6 kb linear plasmid (17). Strikingly, this plasmid migrates to the cytosol in the pollen and is then paternally transmitted to the female mitochondria of the progeny, suggesting that it is able to enter female-inherited mitochondria *in vivo* (22). In the above assays, an artificial expression vector of similar size, the 12.4 kb pBINPLUS plasmid, was not imported into isolated mitochondria (Fig. 1, lane 5). We therefore investigated whether the 11.6 kb mitochondrial plasmid has some particular sequence features which would allow organelle uptake despite its large size.

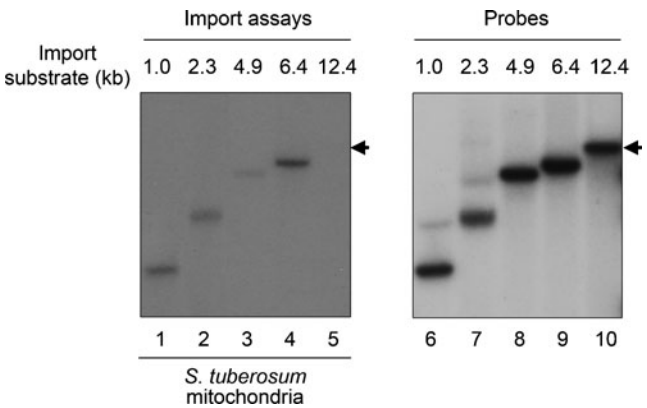

Fig. 1 Size-dependence of DNA import into mitochondria. Left panel: 20 fmoles of labeled DNA substrates of increasing size (see Materials and Methods) were incubated for 45 min at 25°C with isolated potato (*S. tuberosum*) mitochondria in standard conditions (5) prior to DNase-I digestion. Mitochondrial nucleic acids were subsequently extracted, fractionated by agarose gel electrophoresis and transferred onto a nylon membrane which was autoradiographed. Right panel: In parallel with the import assays, 0.1 fmole of the labeled substrates was fractionated by agarose gel electrophoresis and transferred onto a nylon membrane which was autoradiographed. Sizes are indicated in kilo base-pairs (kb). *Arrows* illustrate the absence of import of the linearized 12.4 kb pBINPLUS vector. Images are representative for three independent experiments.

The 11.6 kb plasmid was amplified from *B. rapa* organelle DNA, labeled by PCR and assayed for *in vitro* import into isolated turnip mitochondria. Linearized pBINPLUS was tested in parallel in the same assays. As before, the 11.6 kb and pBINPLUS plasmids were radio-labeled to a similar specific activity, and the same amount of each was used in the import tests. Final analysis showed that whereas the 11.6 kb plasmid was efficiently imported into the organelles, the uptake of pBINPLUS DNA was not detected (Fig. 2a, lanes 1 and 2). To confirm full translocation of the 11.6 kb DNA, a hypotonic shock was applied after the import step, so as to break the outer membrane and generate mitoplasts. DNA not protected by the inner membrane thus became accessible to subsequent DNase I treatment. Incorporation of the 11.6 kb plasmid was resistant to DNAse I treatment of the mitoplasts (Fig. 2b, lanes 3 and 4), implying that the detected signal corresponded to full-size DNA translocated to the matrix side within the organelles. Whereas previous studies supported the idea that DNA uptake was not sequence-specific, at least for relatively small substrates (<5 kb) (5–7), these results, in line with the above experiments, show that the import efficiency of large size DNA clearly depends on the sequence. The *Brassica* 11.6 kb plasmid appears to have such a capacity to be imported into mitochondria despite its large size.

Investigating Specific Sequence Elements in the Import Substrate that can Promote Mitochondrial Uptake

As the regular import substrate is linear DNA, it is possible that the terminal regions may influence the efficiency of the process. A notable feature of the 11.6 kb plasmid is the presence of long (327 bp) terminal inverted repeats. To look closer at the sequence features which may promote import efficiency, the plasmid was amplified so as to exclude the terminal inverted repeats, yielding a 10.9 kb product. The 11.6 kb and 10.9 kb forms (Fig. 2c) were assayed for uptake into isolated *B. rapa* mitochondria. The plasmid without the inverted repeats was no longer recovered in the organelles, while import of the complete form was as before (Fig. 2e, lanes 5 and 6). It thus appeared that the terminal inverted repeats, or at least sequence motifs in them, are indispensable for efficient recognition and translocation of the 11.6 kb plasmid into plant mitochondria. Inside the organelles, these repeats are also believed to bind proteins involved in stabilization and replication of the plasmid (23). Whether such processes contribute to the observed uptake behaviour remains to be investigated.

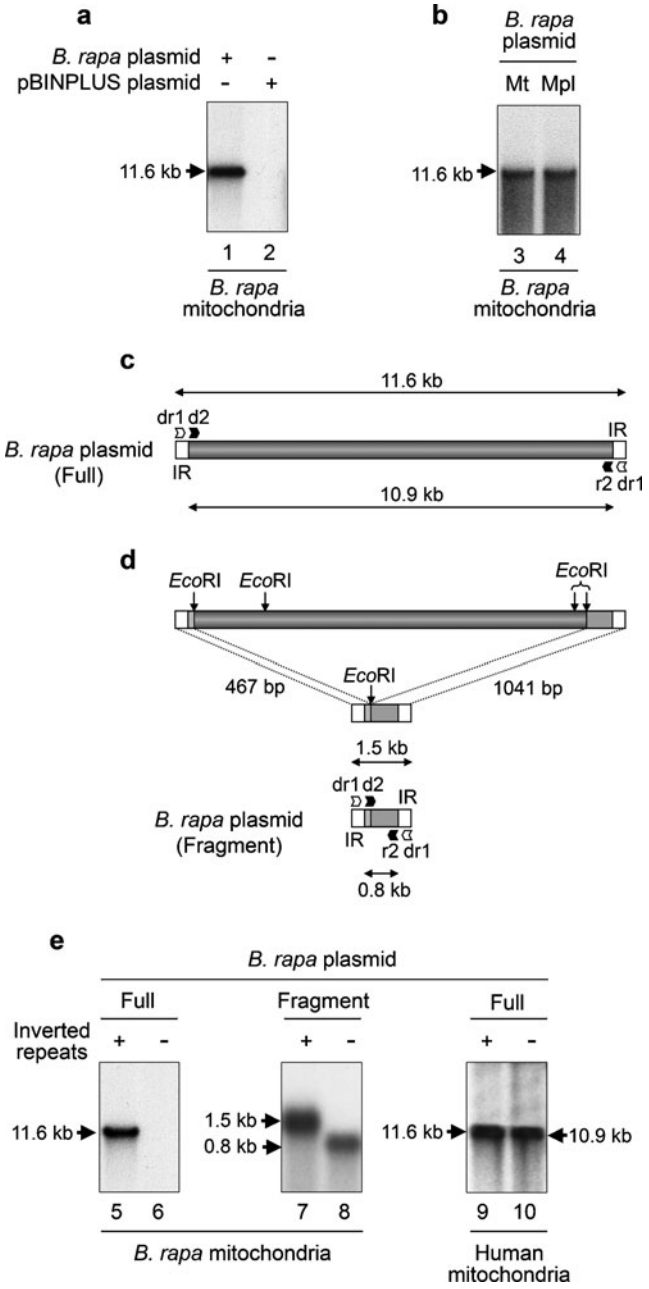

Fig. 2 Import of large size DNA into mitochondria is sequence-dependent. (**a**) Labeled linear turnip (*B. rapa*) 11.6 kb mitochondrial plasmid or linearized 12.4 kb pBINPLUS expression vector were assayed for import into isolated turnip mitochondria in standard conditions. (**b**) Following import of labeled turnip 11.6 kb plasmid, isolated turnip mitochondria were mock-treated (Mt) or submitted to osmotic shock (Mpl) before DNase-I treatment. (**c**) Scheme illustrating the amplification of the full turnip mitochondrial plasmid with (11.6 kb) or without (10.9 kb) the terminal inverted repeats (IR), using the single primer NIBnapIRpl (dr1) or the couple of primers NI10.9A/NI10.9B (d2/r2), respectively. (**d**) Scheme illustrating the deletion of the central part in the cloned turnip mitochondrial plasmid, between the most terminal *Eco*RI sites (see Materials and Methods), and the amplification of the deleted plasmid with (1.5 kb) or without (0.8 kb) the terminal inverted repeats (IR), using the single primer NIBnapIRpl (dr1) or the couple of primers NI10.9A/NI10.9B (d2/r2). (**e**) Import of the full (lanes 5, 6, 9 and 10) or deleted (lanes 7 and 8) labeled turnip mitochondrial plasmid, with or without the terminal inverted repeats, into isolated turnip (lanes 5 to 8) or human (lanes 9 and 10) mitochondria in standard conditions (5,6). Uptake in (**a**), (**b**) and (**e**) was analysed as in Fig. 1. Migration of the incorporated substrates is indicated (11.6 kb, 1.5 kb, 0.8 kb, 10.9 kb). Images are representative for two to five independent experiments, depending on the assay.

To test if the repeats also have an effect on the import of smaller pieces of DNA, a 1.5 kb DNA fragment, in which the two inverted repeats were flanking only a 850 bp internal region of the plasmid, was amplified from an appropriate deletant (Fig. 2d). The same region was also amplified without the inverted repeats. Comparative import assays with the two probes and isolated *B. rapa* mitochondria showed only a very limited increase in the uptake efficiency when the DNA was carrying the inverted repeats (Fig. 2e, lanes 7 and 8). The latter thus seem principally to promote the uptake of larger size DNA. These observations in turn confirm the importance of the sequence content for the import of long substrates into plant mitochondria.

The 11.6 kb *B. rapa* plasmid was further used as a substrate for import into mitochondria isolated from the human HepG2 cell line. Interestingly, this plant plasmid, which is three quarters the size of the whole mammalian mitochondrial DNA (16.5 kb), was also efficiently translocated into human mitochondria (Fig. 2e). Even more remarkably, the 327 bp terminal inverted repeats were dispensable for efficient uptake (Fig 2e, lanes 9 and 10), suggesting that import into human mitochondria has less size restriction.

The 2.3 kb *zea mays* mitochondrial plasmid which served as a substrate in the above experiments (Fig. 1) and in previous studies (5,7) also possesses terminal inverted repeats (15). These are shorter (170 bp) and their sequence differs from that of the 327 bp repeats in the 11.6 kb plasmid. The 2.3 kb substrate was in turn amplified with or without the terminal repeats (Fig. 3a) and assayed in comparative import experiments. The uptake efficiency was not significantly affected by the presence or absence of the repeats, whether considering turnip (Fig. 3b, lanes 1 and 2) or human (Fig. 3b, lanes 3 and 4) mitochondria. This result demonstrated that not all of the various terminal inverted repeats found in linear mitochondrial plasmids are import enhancers. However, it is possible that no effect is detected in these experiments because there is no real sequence dependence of mitochondrial uptake in the size range of the 2.3 kb substrate.

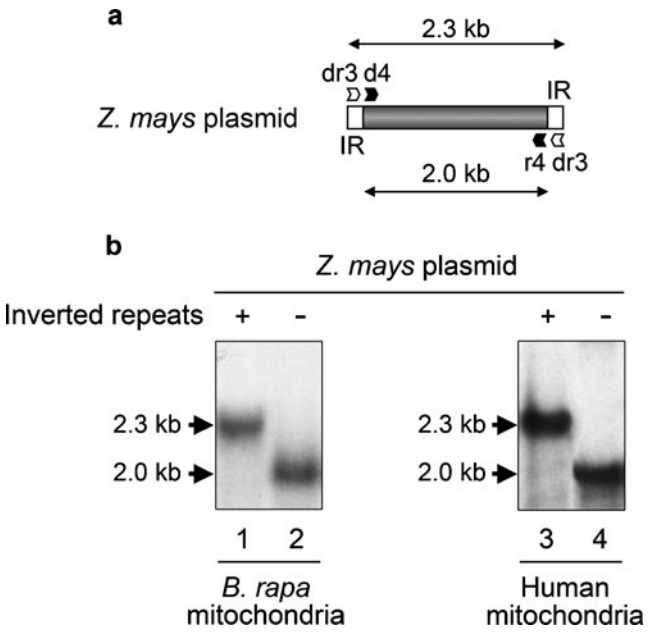

Fig. 3 Stimulation of organelle import is not a general property of terminal inverted repeats from mitochondrial plasmids. (**a**) Scheme illustrating the amplification of the maize (*Z. mays*) mitochondrial plasmid with (2.3 kb) or without (2.0 kb) the terminal inverted repeats (IR), using the single primer NI2.3 kb (dr3) or the couple of primers FL2.3int1/FL2.3int2 (d4/r4), respectively. (**b**) Import of the labeled maize mitochondrial plasmid, with or without the terminal inverted repeats, into isolated turnip (lanes 1 and 2) or human (lanes 3 and 4) mitochondria in standard conditions (5,6). Uptake was analysed as in Fig. 1. Migration of the incorporated substrates is indicated (2.3 kb, 2.0 kb). Images are representative of two independent experiments.

DNA Import into Mitochondria Can Be Enhanced by ATP

The mechanism of translocation through the double mitochondrial membrane is not well understood, but whether considering plant, mammalian or yeast mitochondria, DNA import is obtained in a minimal medium containing an osmoticum and a buffer (5–7). These conditions were sufficient for previous functional studies (5,6,9,10). However, effectors like CCCP or oligomycin, which indirectly or directly affect ATP production, influence DNA import (5). We have thus introduced ATP into import assays using as a substrate the 2.3 kb *zea mays* mitochondrial plasmid. Addition of millimolar concentrations of ATP indeed stimulated DNA import into isolated human mitochondria (Fig. 4a). Strikingly, not only the uptake was enhanced but the imported DNA co-migrated with the resident mitochondrial DNA. This suggests that when ATP is provided in the medium, the uptake mechanism allows a tighter integration into the mitochondrial nucleoids. Despite extensive proteinase K treatment of the final samples (see Materials and Methods), the exogenous DNA might subsequently remain associated with the mtDNA in our extraction conditions (Fig. 4a, lane 2), possibly through unreleased nucleoid factors. Addition of ATP, with or without $MgCl_2$, also stimulated DNA import into *S. cerevisiae* mitochondria (Fig. 4b, lanes 5 and 6), and further investigations were developed with the yeast model system.

The fate of the ATP in the import assays was thus analyzed. For that, yeast mitochondria were incubated for different times in import buffer containing [α-^{32}P]ATP and re-isolated by centrifugation through a sucrose cushion. The original reaction medium (remaining on top of the cushion) and the content of the mitochondrial pellets were fractionated by thin layer chromatography on PEI-cellulose plates. Most of the radioactivity was recovered in the reaction medium, with the whole of the initial labeled ATP being converted into ADP and AMP already after 15 min (Fig. 4c, lane 8). ATP thus appeared to be rapidly hydrolyzed in DNA import conditions. However, labeled ATP was never detected in the mitochondrial fraction, which displayed only ADP and AMP upon TLC analysis (Fig 4c, lanes 10 to 12). Unless hydrolysis inside the organelles was extremely rapid, these observations suggested that the ATP added to the medium was converted on the outside of mitochondria. To determine whether this hydrolysis was important to enhance DNA import, mitochondria were pre-incubated with unlabeled ATP for 15 min, washed with import buffer and used for DNA uptake assays without further addition of ATP. In such conditions, DNA import was not enhanced, as compared to assays run without pre-treatment of the organelles. It thus appeared that stimulation of DNA transport across the mitochondrial membranes needed the hydrolysis of ATP to occur during the uptake process. Finally, further experiments showed that also AMP increased DNA import (Fig. 4d, lanes 15 and 16). However, upon uptake assays run with [α-^{32}P]AMP, it turned out that the latter was incorporated into the organelles at a very high rate, subsequently leading to an efficient release of labeled ATP into the medium (Fig. 4e). Thus, AMP stimulation of DNA import might actually result from rapid ATP production and release.

Protein Phosphorylation May Be Related to Mitochondrial Competence

Mitochondrial proteins can be phosphorylated upon incubation with ATP (24,25), which might be the case in our assays. To test this possibility, yeast mitochondria were purified on sucrose gradient, incubated for 30 min in import buffer containing [γ-^{32}P]ATP and washed. Membrane proteins were then extracted, separated by SDS-PAGE and analysed by autoradiography. As shown in Fig. 5a, several proteins were radioactively labeled during

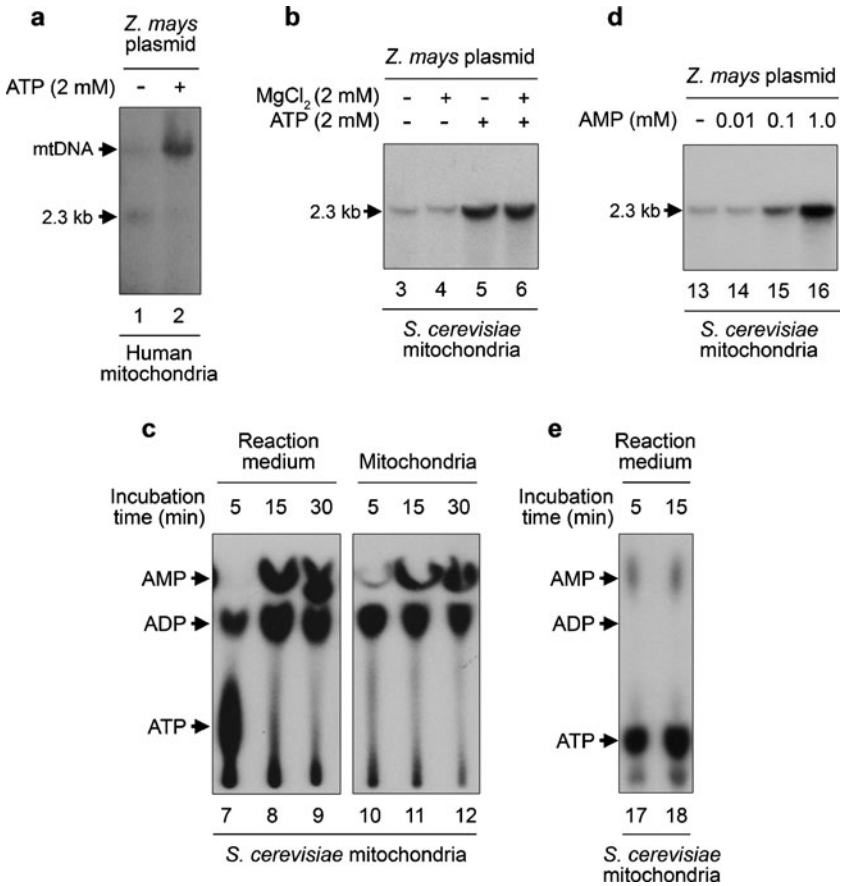

Fig. 4 DNA import into mitochondria is enhanced by ATP. (**a**) Import of the labeled maize 2.3 kb mitochondrial plasmid into isolated human mitochondria in standard conditions (6) without or with addition of ATP in the reaction medium. (**b**) Import of the labeled maize 2.3 kb mitochondrial plasmid into isolated yeast (*S. cerevisiae*) mitochondria in standard conditions (7) without or with addition of $MgCl_2$ and/or ATP in the reaction medium. (**c**) Thin layer chromatography analysis of the nucleotides in the reaction medium and the organelle fraction after incubation of yeast mitochondria for different times in import buffer containing [α-^{32}P]ATP. Chromatography was developed in formic acid/LiCl solvent. Labeled nucleotides were revealed by autoradiography. (**d**) Import of the labeled maize 2.3 kb mitochondrial plasmid into isolated yeast mitochondria in standard conditions with increasing concentrations of AMP in the reaction medium. (**e**) Thin layer chromatography analysis of the nucleotides in the reaction medium after incubation of yeast mitochondria for different times in import buffer containing [α-^{32}P]AMP. Chromatography was developed in isobutyric acid/ammonia solvent. Labeled nucleotides were revealed by autoradiography. DNA uptake in (**a**), (**b**) and (**d**) was analyzed as in Fig. 1. Migration of the mitochondrial DNA (mtDNA) and of the incorporated DNA substrate (2.3 kb) is indicated. In (**c**) and (**e**), migration of unlabeled AMP, ADP and ATP, as detected under UV light, is marked. Images are representative of three to five independent experiments, depending on the assay.

the assay, essentially in the range of 55 to 65 kDa. Phosphorylation of membrane proteins in that range was also detected by specific fluorescent staining with Pro-Q Diamond reagent after incubation of *S. cerevisiae* mitochondria in DNA import conditions with or without non-radioactive ATP. Pro-Q Diamond fluorescent stain (Molecular Probes) allows direct, in-gel detection of phosphate groups attached to tyrosine, serine, or threonine residues. Incubation with ATP led to increased phosphorylation of two main polypeptides, but no protein was newly phosphorylated (Fig. 5b, lanes 15 and 18). The relevance of protein phosphorylation for the mechanism of DNA import was tested by using phosphatase inhibitors during organelle isolation. Supporting such a hypothesis, *S. cerevisiae* mitochondria prepared in the presence of phosphatase inhibitors showed a much higher DNA uptake capacity than organelles isolated without inhibitors (Fig. 5c).

DISCUSSION

Natural competence of isolated mitochondria has been firmly established through direct evidence of DNA internalization and through functional analyses (5–10) (Table 1). Mammalian mitochondria show more flexibility, as they accept single-stranded DNA in addition to double-stranded substrates (6). From a mechanistic point of view, the involvement of the VDAC in DNA translocation through the outer membrane appears to be common to plant, mammalian (including human) and *S.*

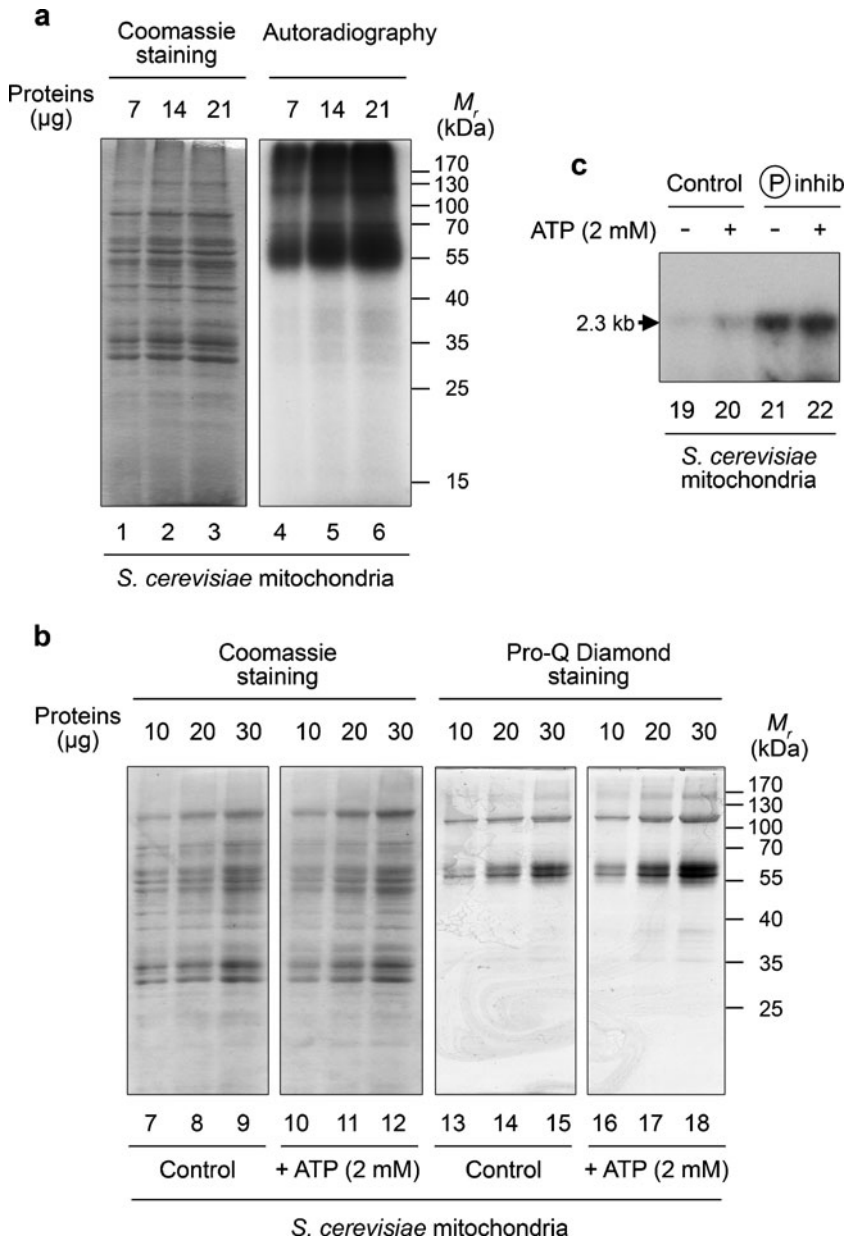

Fig. 5 Mitochondrial protein phosphorylation and DNA import. (**a**) SDS-PAGE analysis of membrane proteins after incubation of yeast mitochondria in import buffer containing [γ-^{32}P] ATP. Three increasing protein amounts were loaded on gel. The protein pattern was revealed by Coomassie Blue staining. Protein phosphorylation was dectected by autoradiography. (**b**) SDS-PAGE analysis of membrane proteins after incubation of yeast mitochondria in import buffer without or with unlabeled ATP. Three increasing protein amounts were loaded. The protein pattern was revealed by Coomassie Blue staining. Protein phosphorylation was detected by Pro-Q Diamond staining. (**c**) Import of the labeled maize 2.3 kb mitochondrial plasmid into yeast mitochondria isolated in the absence or presence of phosphatase inhibitors [(P) inhib]. Assays were run without or with addition of ATP in the reaction medium. In (**a**) and (**b**), migration of size marker proteins is indicated on the right side of the panels [(M_r (kDa)]. DNA uptake in (**c**) was analyzed as in Fig. 1. Migration of the incorporated DNA substrate is indicated (2.3 kb). Images are representative of two independent experiments.

cerevisiae mitochondria (5–7). Conversely, how the DNA crosses the inner membrane is still an open question, and this step may somehow differ between organisms (Table 1). DNA uptake into plant organelles is sensitive to a number of effectors, in particular substrates or inhibitors of the adenine nucleotide translocator (ANT) (5). By contrast, most of these effectors, including uncouplers, have no significant effect on DNA import into mammalian mitochondria. In that case, translocation through a pore, rather than involvement of nucleotide transporters, has been suggested (6). Finally, the contribution of respiratory substrates to the efficiency of DNA uptake is also common to plant, mammalian and yeast mitochondria but remains modest.

Early experiments demonstrated *in organello* transcription of the *gfp* sequence driven by the promoter of the mitochondrial 18S rRNA gene upon import of the corresponding construct into plant (*S. tuberosum*) organelles (5). Synthesis and subsequent processing of a tRNA precursor were also obtained in this system (8). Similarly, two marker genes (*gfp* and luciferase) were transcribed in rat liver mitochondria from an imported construct (6). Transcription was under the control of the rat mtDNA heavy-strand or light-strand promoter (HSP1/LSP). In these assays, the *gfp* sequence was expressed *in organello* as a co-transcript with the human mitochondrial $tRNA^{Arg}$. The latter was shown to be processed from the co-transcript and fully matured, including 3′-CCA addition. In both plant

Table I Mechanistic and Functional Data from DNA Import Assays with Plant, Mammalian and Yeast Mitochondria

Source of mitochondria	Plant	Mammal	Yeast
Appropriate DNA substrate	ds linear[a]	ds linear; ss linear[b]	ds linear[c]
Involvement of the VDAC in OM translocation	yes[a,c]	yes[b,c]	yes[c]
Effect of respiratory substrates on import	↑[a]	↑[b]	↑[c]
Effect of uncouplers on import	↓[a]	no effect[b]	↓[d]
Involvement of nucleotide carriers in IM translocation	ANT[a]	no[b]	ANT ?[c]
Recombination of the imported DNA	yes[e]	strand invasion ?[b]	nd
Imported DNA as a template for DNA synthesis	yes[a]	yes[b]	nd
Repair of the imported DNA	yes[f]	yes[g]	nd
Transcription of the imported DNA	yes[a,h]	yes[b]	nd
Processing of the transcribed RNA	yes[a,h]	yes[b]	nd

ds double-stranded; *ss* single-stranded; ↑: enhancement of import; ↓: decrease of import; *OM* outer membrane; *IM* inner membrane; *ANT* adenine nucleotide translocator; *nd* not determined. [a] Koulintchenko *et al.*, 2003 (5); [b] Koulintchenko *et al.*, 2006 (6); [c] Weber-Lotfi *et al.*, 2009 (7); [d] Weber-Lotfi *et al.*, unpublished; [e] Mileshina *et al.*, 2011 (11); [f] Boesch *et al.*, 2009 (9); [g] Boesch *et al.*, 2010 (10); [h] Placido *et al.*, 2005 (8)

and mammalian organelles, the imported DNA acts as a template for DNA synthesis (5,6). Moreover, uptake of uracil-containing DNA provided the first direct evidence for DNA repair in plant mitochondria (9) and enabled characterization of the functional organization of the base excision repair (BER) pathway in mammalian organelles (10). Using as import substrates constructs composed of a partial *gfp* gene flanked by fragments of mtDNA led to *in organello* homologous recombination with the resident DNA and integration of the reporter gene in *S. tuberosum* and *Nicotiana tabacum* mitochondria (11). In mammalian organelles, imported DNA carrying mtDNA sequences also associates with the resident genomic DNA, although regular recombination has not been established so far (6).

Based on these robust data, it can be hypothesized that organelle competence has the potential to support mitochondrial transfection in whole cells. *In vivo* occurrence of such processes is not established as such but can be proposed, for instance, for the distribution of mitochondrial plasmids. As mentioned above, previous studies in *B. napus* showed that the 11.6 kb plasmid used as an import substrate in the present work migrates to the cytosol in sperm cells of mature pollen, whereas in parallel the main mitochondrial DNA disappears (22). Nevertheless, the plasmid is subsequently recovered in the organelles of the progeny, suggesting that it has been taken up by the female-inherited mitochondria.

With such a view, mitochondrial transfection becomes a matter of carrying an appropriate substrate DNA into the cells and to the vicinity of the organelles in the right physiological context. Different types of mitochondriotropic nanocarriers able to bind DNA and enter mammalian cells have been developed (26). These include vesicles made of dequalinium, so-called DQAsomes (27), and mitochondriotropic liposomes (28). Both types of carrier drive DNA into mammalian cells and release their load when they meet mitochondria (28,29). The DNA subsequently remains associated with the organelles, which raises the hypothesis that it can be taken up through the import pathway that we established with isolated mitochondria. Interestingly, organelle co-localization was more efficient with linear DNA than with a circular substrate (30), which fits the inability of isolated mitochondria to incorporate circular DNA (5).

Another option to transfer exogenous DNA could be to import the substrate of interest into isolated mitochondria and introduce the transfected organelles into recipient cells. Following early observations (31,32), Katrangi *et al.* (12) recently described supporting evidence for a potentially inherent ability of mammalian cells to internalize isolated mitochondria. On the other hand, microinjection of isolated organelles into mammalian cells has been used to establish model systems or to analyze embryo development (33–35). Obviously, the suitability of organelle internalization or microinjection to manipulate mitochondrial genetics in cells depends on efficient *in vitro* transfection of the original isolated organelles.

The present work brings further critical information for all these approaches. So far, import experiments with plant, mammalian or yeast organelles and DNA substrates in the range of 0.5 to 4 kb inferred that DNA uptake into mitochondria was sequence-independent. We show here that larger size DNA up to 12 kb can be translocated into the organelles, but this occurs on a sequence-selective basis. Both *in vitro* loading of isolated mitochondria and nanocarrier-mediated *in vivo* transfection with larger size DNA carrying functions of interest will therefore require DNA substrates to be carefully designed, so as to optimize their import. A remarkable result in this respect was the efficient uptake of the 11.6 kb turnip plasmid, contrasting with an undetectable import of the similarly sized pBIN-

PLUS vector in five independent experiments. Even more striking, at least in the case of plant mitochondria, uptake of the turnip plasmid was driven by its terminal inverted repeats. These features support the existence of one or several recognition step(s) during the process, and the inverted repeats of the turnip plasmid might in turn be suitable to improve future originally inefficient import substrates. On the other hand, our results showing that human mitochondria efficiently imported the turnip plasmid deprived of its terminal repeats suggest that the size threshold from which specific sequences are required for uptake is higher in that case. The data thus open the prospect of targeting whole mitochondrial genomes into mammalian organelles *in vitro* and *in vivo*, or complex constructs potentially competent for replication. The latter aspect is of primary importance for nanocarrier-mediated mitochondrial delivery of therapeutic DNA, which will be restricted by the toxicity of the carrier and will benefit from constructs able to be maintained in the transfected organelles.

A further important functional aspect of DNA import into mitochondria, as highlighted in our assays, is the contribution of ATP. Remarkably, experiments with human organelles suggest that ATP not only enhances uptake but also influences the sub-mitochondrial integration of the exogenous DNA. The genetic material in mitochondria is packed into functional nucleoprotein particles which are referred to as the nucleoids and where DNA metabolism is taking place (36). The presence of ATP in the assays seems to be associated with a better integration of the exogenous DNA into these active structures, an issue which is of particular relevance for therapeutic DNA. The putative involvement of co-factor phosphorylation in these processes needs to be further documented. For assays run with yeast organelles, preliminary mass spectrometry identification of the proteins located in the areas stained with Pro-Q Diamond in the gel presented in Fig. 5b (lane 18) included mitochondrial HSP60. Indeed, known to be phosphorylated, this chaperonin is primarily considered to be involved in protein transport and folding. Interestingly, it also binds DNA and plays a role in nucleoid structure and transmission (37). Tight integration of the exogenous DNA into the organelle genetic processes might have an additional functional advantage. Recent results confirm the occurrence of limited homology-dependent recombination in mammalian mitochondria, especially for double-strand break repair (38,39). Imported DNA with a proper sequence content might thus promote recombination with the resident mtDNA.

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