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Mitochondrial gene therapy: an arena for the biomedical use of inteins

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Mitochondrial DNA (mtDNA) mutations underlie many rare diseases and might also contribute to human aging. Gene therapy is a tempting future possibility for intervening in mitochondrialopathies. Nuclear expression of the 13 mtDNA-encoded proteins might be the most effective gene-therapy strategy. Its only confirmed difficulty is the extreme hydrophobicity of these proteins, which prevents their import into mitochondria from the cytosol. Inteins (self-splicing 'protein introns') might offer a solution to this problem: their insertion into such transgenes could greatly reduce the encoded proteins' hydrophobicity, enabling import, with post-import excision restoring the natural amino acid sequence.

The human mitochondrial DNA (mtDNA) is a circular genome of 16 569 bp that encodes 13 proteins and the RNA components of the machinery for their translation (two ribosomal RNAs and 22 transfer RNAs). Because all 13 proteins are subunits of the enzymes that perform oxidative phosphorylation, loss-of-function mutations in any part of the mtDNA deprive the cell of most of its ATP-synthesis capacity. Since 1988, many mtDNA mutations have been discovered that cause neural and/or muscular dysfunction¹. Many are inherited, but others occur sporadically and are presumably caused by spontaneous mutations early in development, or even in the unfertilized oocyte.

The accumulation of spontaneously mutant mtDNA might also play a role in normal aging^{2,3}. Although the quantity of mutant mtDNA that elderly humans possess is widely thought to be much less than in mitochondrialopathies⁴, its presence in the body for a normal lifetime has the potential greatly to amplify its effects, perhaps via the extracellular production of toxic free radicals by the rare cells that lack any wild-type mtDNA^{5,6}.

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Progress in the development of therapies has not been encouraging in either aging or mitochondrialopathies. This strongly motivates the consideration of innovative interventions that differ qualitatively from those that are currently available. Of these, the most promising might be gene therapy, because most of the technological breakthroughs that are needed to introduce engineered DNA into somatic cells are the same for any such DNA and so are being worked on by large numbers of research groups whose interests are not related to mtDNA mutations.

However, gene therapy of mtDNA mutations also faces a specific difficulty in addition to those that apply to gene therapy of nuclear DNA mutations. Either the engineered DNA must be delivered to a cellular location that traditional vectors do not target (the mitochondrion) or its products must be transported to its natural location in the mitochondrion despite being synthesized in a different compartment (the nucleus in the case of RNAs, the cytosol in the case of proteins). Fortunately, we need not wait for the gene-therapy breakthroughs before addressing these mtDNA-specific problems because they can be explored first *in vitro* and then using germ-line transformation in mice. Three strategies have been advocated for mitochondrial gene

therapy, which address this problem in different ways (Fig. 1): (1) to inhibit the ability of the mutant mtDNA to replicate; (2) to express replacement proteins from transgenic DNA targeted to the mitochondria; and (3) to express modified replacement proteins from transgenic DNA targeted to the nucleus.

General strategies for mitochondrial gene therapy

An approach that might hold great promise for the rare mitochondrial pathies is the mitochondrial targeting of antisense oligonucleotides that bind specifically to mutant mtDNA sequences and prevent their replication, thereby allowing any wild-type mtDNA present in the same cell to increase in copy number⁷ (Fig. 1a). However, this approach is not likely to be useful against aging, in which many different mtDNA mutations arise in different cells. Furthermore, it also risks promoting mtDNA depletion and the creation of mitochondria with no mtDNA.

An alternative (Fig. 1b) is to introduce replacement DNA into the mitochondria; this would genetically complement the mutant mtDNA (or, in a variation of this idea¹⁸, repair it by homologous recombination) rather than eliminating it⁸. This avoids the problem of mtDNA depletion but it is not clear whether or not the endogenous and highly evolved mitochondrial transcription and translation systems (which are specialized for the expression of a single circular DNA molecule) would operate on the, presumably very differently structured, DNA fragments that would be introduced in this way.

Both of these pathways also face the difficulty of targeting nucleic acids to the mitochondria. It seems that at least two RNA species are imported into the human mitochondria^{9,10} but the mechanism is not well understood. By contrast, we now have fairly detailed knowledge about the mitochondrial import of proteins by the TIM-TOM complex¹¹ (translocases of the inner and outer membrane, respectively). One possibility is that nucleic acids might be imported by the protein-import machinery if they were covalently bound to oligopeptide presequences¹²; this has also been proposed as a delivery technique for mitochondrial gene therapy^{7,8}. However, until more information is available about how RNAs are naturally imported into human mitochondria, one must be cautious about the biomedical applicability of this process.

The third option (Fig. 1c), which was first advocated in 1990¹³, is to target replacement mtDNA sequences not to the mitochondria but to the nucleus, having first modified them in two ways. The first modification is making base-pair substitutions to compensate for the differences between the human nuclear and mitochondrial genetic codes. This means that the transgenes will encode the correct amino acid sequences even though they will be translated in the cytosol. The second change is adding a presequence to target the protein to the mitochondria. This pathway is called allotopic expression and is ostensibly the most promising intervention because it only uses well-characterized cellular machinery (nuclear gene expression and mitochondrial protein import) and the same gene-delivery technology that is being extensively pursued for the gene therapy of nuclear DNA mutations. A 'proof of

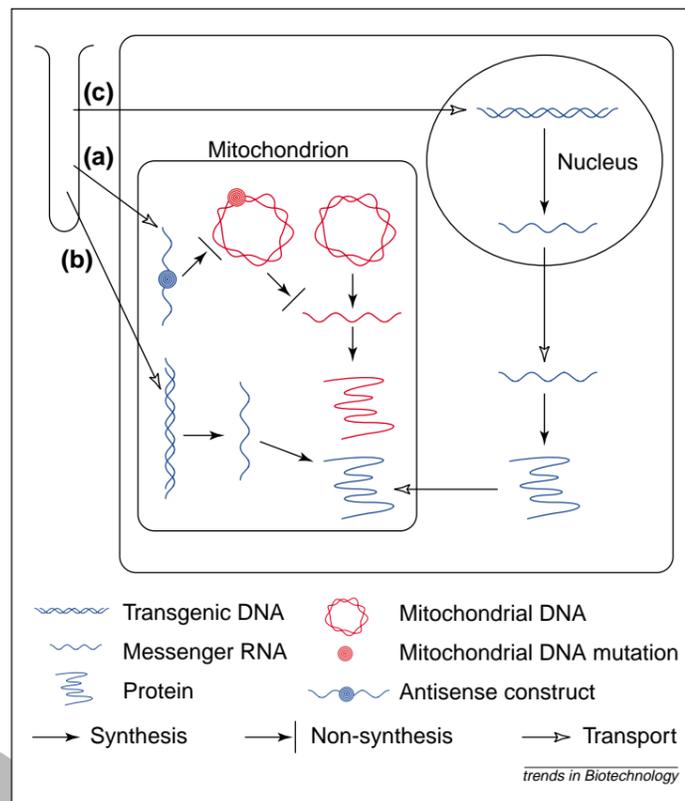


Figure 1

Three approaches to mitochondrial gene therapy. (a) Antisense-mediated inhibition of the replication of mutant mtDNA. (b) Introduction of replacement mtDNA into the mitochondria. (c) Introduction of modified replacement DNA into the nucleus, whose protein products would be imported into mitochondria. Red represents endogenous molecules and blue represents transgenic constructs and their products.

concept' of this approach has existed since 1988, when Nagley *et al.* demonstrated the phenotypic 'rescue' of a mutation in yeast ATPase subunit eight by a suitably recoded, plasmid-borne (nuclear) transgene¹⁴. It has since also been proposed as an antiaging therapy^{15,16}.

Challenges to the usefulness of allotopic expression

Despite the early successes described above, there has been little enthusiasm for allotopic expression in recent years. Although several objections have been raised, most do not appear, on closer inspection, to be justified. A surprisingly frequent suggestion^{7,17,18} is that the allotopic expression of the mitochondrion-encoded (mt-coded) proteins will not combat mutations in the mtDNA genes encoding RNAs (which are frequent in both aging and mitochondrial pathies). However, this overlooks the fact that the mt-coded RNAs have only one known function: to participate in the synthesis of the 13 proteins that (under the allotopic-expression scheme) would be imported from the cytosol. They are thus superfluous once all those proteins are being imported.

It has also been suggested that the mt-coded proteins might be toxic to some cytosolic processes¹⁹ or might be targeted to the endoplasmic reticulum and secreted before reaching the mitochondria²⁰. However, there is negligible evidence for either phenomenon, and the

original arguments were based more on the need to explain why the transfer of genes from the mtDNA to the nucleus during evolution has stopped with 13 such genes still untransferred. This observation can in fact be explained more persuasively by other hypotheses that also explain why many of these genes have been transferred in various plant taxa²¹ (see below).

Another objection is that the transgenes must be expressed only when mitochondrial biogenesis is required, and this regulation is as yet only poorly understood. However, there is good reason to predict that appropriate regulation can be achieved even without this knowledge, because all 13 mt-coded proteins are subunits of enzymes that also have nucleus-encoded subunits in 1:1 stoichiometry. The regulatory sequences surrounding those nucleus-encoded genes can be used in the transgenic constructs and should, therefore, suffice to ensure the appropriate regulation of the transgenes.

A challenge that might be more justified, but that is also not yet supported by any firm evidence, is based on stoichiometry. All 13 mt-coded proteins are subunits of extremely intricate enzyme complexes that also contain many nucleus-encoded subunits. Thus, if a mitochondrion is importing an inappropriate quantity of a particular subunit then the assembly of the complex might be disrupted. This problem is further complicated by the fact that some mitochondria might still be expressing normal quantities of the subunit from the endogenous mtDNA but some will not, and some might even be expressing mutant forms of the subunit.

However, it seems premature to allow such logic to stand in our way because it is easy to contrive ways to combat this problem if it arises, such as blocking mtDNA replication, transcription or translation by introducing transgenes encoding DNA- or RNA-binding proteins that would block the progress of polymerases or ribosomes. As such binding need not even be sequence specific, this approach is technologically straightforward except for the gene-delivery process, which is assumed to be available by the time this becomes relevant.

A further potential difficulty arises from the fact that import entails unfolding these proteins in order to thread them through the TIM-TOM machinery¹¹, and their subsequent refolding might not occur correctly. No evidence on this point is yet available, however, and the highly versatile chaperones that mediate the folding of proteins emerging from a mitochondrial ribosome²¹ might also work efficiently on the same proteins emerging from the TIM complex.

The unimportability of highly hydrophobic proteins

There is one undisputed obstacle to achieving allotropic expression, however – the mt-coded proteins are very hydrophobic and are thus highly resistant to the unfolding necessary for import. This has been recognized for a long time. The definitive study identified a measure of hydrophobicity and, according to this, all the *Saccharomyces cerevisiae* mt-coded proteins rank above any known imported proteins²². Several imaginative approaches to combating this difficulty have been proposed, some of which have been investigated experimentally.

The first was to attach an extremely long leader sequence²³, which might allow the import machinery to apply greater transmembrane force to the protein being imported. This was motivated by the observation that highly hydrophobic nucleus-encoded proteins tend to have unusually long leader sequences. This succeeded only modestly, however: the 76-amino-acid subunit nine of *S. cerevisiae* ATPase was successfully imported but longer proteins were not²².

Two other suggested approaches are based on the observations that protein import begins at the N terminus and so can, in principle, begin while translation is still occurring, and that this co-translational import often actually happens²⁴. The relevance of this is that, if import can 'keep pace' with translation, hydrophobic domains can be imported before they have time to fold. Thus, the goal is to make import more co-translational. This might be achieved by slowing down the translation of the allotopically expressed transgenes or by accelerating the transport of their mRNAs to the vicinity of the mitochondria that are engaged in import. The former could be done by synonymous substitutions, introducing many rare codons (a manipulation that can vary bacterial protein-synthesis rates by a factor of six²⁵), but this has not yet been attempted. The latter appears to be more ambitious but might be the mode of action of a karyopherin whose multicopy expression in yeast allows the import of otherwise unimportable sections of cytochrome b (Ref. 26).

Another plausible approach is to investigate how these proteins are imported in other organisms. The variant genetic code of all animal species' mtDNA is an absolute prohibition on further gene transfer to the nucleus during evolution. The barrier derives mainly from the earliest deviation: the change in meaning of the UGA codon from STOP to tryptophan²¹. This occurred contemporaneously with the cessation of gene transfer in animals and fungi, so no other explanation for that cessation need be sought. Various plant taxa, in which the mtDNA still uses the standard genetic code, have indeed successfully transferred to the nucleus genes that are still encoded mitochondrially²⁷⁻²⁹. Nothing is yet known, however, about how the hydrophobicity problem has been overcome.

Dispensing with Complex I

The difficulty facing all the above approaches is that they are only quantitative improvements to the existing system, in that they raise, but do not remove, the hydrophobicity barrier to import. Thus, they might allow the import of some of the offending 13 proteins but are unlikely to suffice for the most hydrophobic proteins. The limits of improvement that can be conferred by long leader sequences are already apparent²².

An alternative is to import different, less hydrophobic, proteins that would functionally substitute for the unimportable ones. This might already be possible for Complex I. Some yeast taxa have dispensed with Complex I in favour of a single-polypeptide enzyme that performs the same electron-transfer function but does not pump protons; for proton-pumping, the capacity of Complexes III and IV is sufficient³⁰. This non-proton-pumping NADH dehydrogenase is not excessively

hydrophobic and is already nucleus-encoded. Seo *et al.* introduced a transgenic copy from *S. cerevisiae* into Chinese hamster cells that were mutant for a vital Complex I subunit and showed that it could sustain them when grown on galactose, which is an inefficient source of ATP from glycolysis and did not support the growth of non-transgenic, Complex-I-deficient cells³¹. They later showed that human cells could be similarly modified and that the normal Complex I could coexist with the *S. cerevisiae* protein without the function of either protein being inhibited³².

However, this approach has severe limitations. First, the proton-pumping capacity of Complexes III and IV, although adequate in culture, might not suffice *in vivo*; this might soon be tested in mice³². Second, there is the inescapable problem that the concept can only be applied to Complex I and Complexes III, IV and V also have some mt-coded subunits. Because most somatic mtDNA mutations seem to be large deletions or tRNA mutations (which would eliminate all 13 proteins), the effect of a Complex-I-specific therapy might be rather small.

It is possible that the alternative oxidase of plants, which transfers electrons directly from ubiquinol to oxygen and thus bypasses both Complexes III and IV (Ref. 33), could do for those complexes what the yeast NADH dehydrogenase does for Complex I. However, they clearly cannot both be introduced together, because then all three proton-pumping enzymes of the electron-transport chain would be bypassed and no proton pumping would occur. Furthermore, the genes for two of the mt-coded subunits (cytochrome b of Complex III and subunit I of Complex IV) are present in every mitochondrial genome yet sequenced, indicating that their encoded proteins' hydrophobicity has made those genes' transfer to the nucleus evolutionarily impossible even in taxa that lack a disparity of genetic codes. Accordingly, even a combination of all the strategies explored to date might fail to achieve complete allotopic expression of the mtDNA.

Inteins: a potentially universal solution

There is one more theoretical possibility: the construction and import of less hydrophobic proteins followed by their post-import modification into replicas of the normally mt-coded proteins. Such a strategy undoubtedly seems daunting at first sight but there is a technology that might make it relatively feasible. Inteins³⁴, which were first discovered in 1990, are self-splicing 'introns of proteins'. They excise post-translationally from the protein, ligating the 'exteins' on either side with a bona fide peptide bond (Fig. 2a). Most have been found in Bacteria and Archaea but six are known to exist in eukaryotes, including two in the nuclear genome of yeasts³⁵. 'Split inteins' have also been discovered³⁶, in which the two exteins are initially encoded on separate mRNAs but nonetheless ligate to give a single polypeptide (Fig. 2b).

From a biotechnology perspective, a particularly attractive feature of inteins is that autosplicing does not require specific extein consensus sequences – the only absolute requirement is that the amino acid that is immediately C-terminal to the intein must be a cysteine, a serine or a threonine (Fig. 2). The endopep-

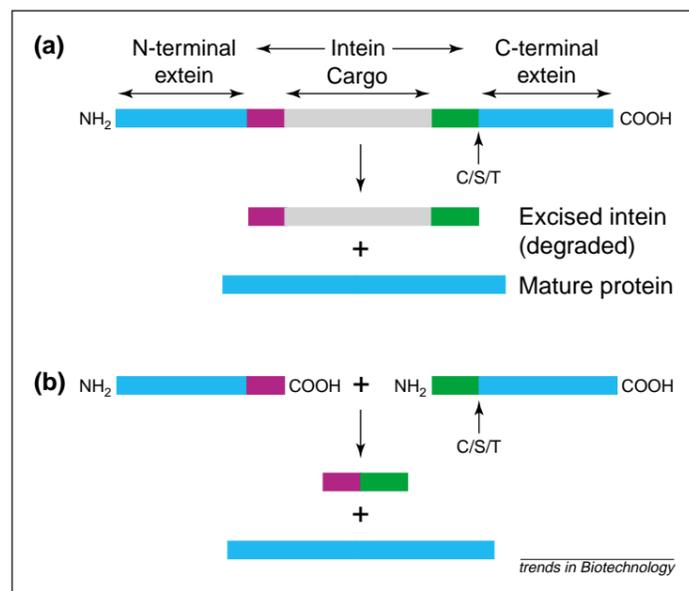


Figure 2

Structure and maturation of inteins. (a) The prototypical intein structure, including a cargo (grey) between the active endopeptidase sequences (mauve and green). (b) A split intein, encoded on two separate transcription units, whose protein products ligate to form a functional intein that then excises.

tidase sequences of inteins are restricted to several dozen amino acids at either end; many inteins contain several hundred amino acids of 'cargo' between these sequences, which is superfluous for intein maturation (although it typically has intriguing endonuclease activity)³⁴.

Two potential uses of inteins to facilitate allotopic expression are illustrated in Fig. 3. It has been calculated that import is, on average, much faster than translation³⁷. Thus, increasing the spacing between a protein's hydrophobic domains might ease its import by importing the long hydrophilic region faster than translation is proceeding (i.e. by allowing cotranslational

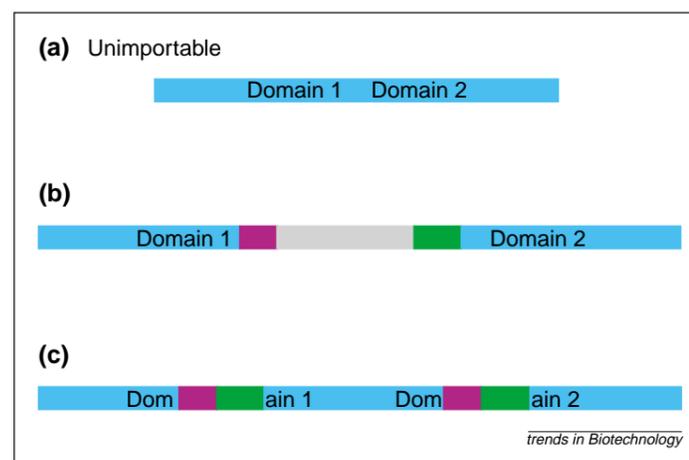


Figure 3

Two methods by which inteins might improve the mitochondrial import of highly hydrophobic proteins (a). (b) The hydrophobic domains are moved further apart, allowing their import to be more cotranslational. (c) The hydrophobic domains are bisected by inteins, so that they cannot fold.

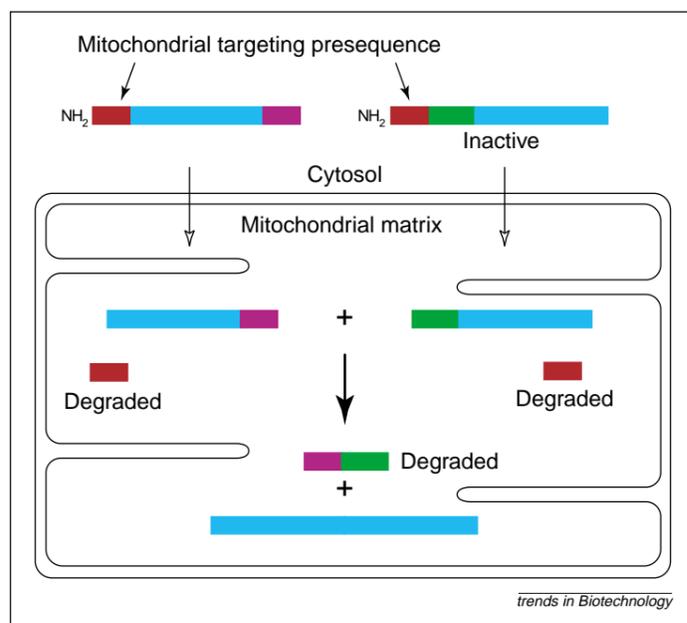


Figure 4

A model for reliable compartment-specific maturation of split inteins. The C-terminal extein is inactivated while in the cytosol by the mitochondrial targeting sequence. When this is removed after import, maturation can proceed.

import to catch up with translation). Supporting this concept, it has been shown that hydrophobic domains impede import less when they are isolated than when they are close together²². Alternatively, and perhaps more powerfully, inteins might be inserted within hydrophobic domains, and this would prevent those domains from folding (and inhibiting import). Excised inteins should be rapidly destroyed by the same mechanism that degrades N-terminal mitochondrial targeting presequences.

Delaying maturation until after import

The major requirement that must be met before inteins can be used for allotropic expression is that they must be prevented from excising while they are still in the cytosol. Natural inteins excise rapidly and autonomously, with no cofactors or chaperones, and so they will need modification. A conceptually simple approach is to develop conditional inteins, which would be inactive in the cytosol but would excise once they were in the mitochondrial matrix as a result of its distinctive chemistry (e.g. its pH, which is appreciably higher than that of the cytosol). Several groups have reported pH-sensitive inteins^{38–40}, some of them optimal at the intramitochondrial pH⁴⁰. There would presumably be some value in maximizing the compartment specificity of the inteins and so a mutagenesis approach using strong selection for this feature might be appropriate; indeed, this method of intein optimization has already met with success³⁸. A promising alternative is related to the idea of the wide spacing of hydrophobic domains. If an intein is long enough, and if import is engineered to be predominantly co-translational, it should be possible to have the intein's N terminus imported before its C terminus has been constructed. If so, excision could not occur until the whole intein had been imported.

Delayed maturation of split inteins

However, neither of the above strategies seems likely to produce strictly compartment-specific excision. This is a serious drawback because proteins that resulted from the cytosolic excision of the inteins would not necessarily be rapidly degraded; more probably, they would be targeted by the mitochondrial protein-import machinery and become stuck in a TIM-TOM complex at the first prohibitively hydrophobic domain. This would inactivate the TIM-TOM complex unless and until the protein is degraded from either side, a process that has not yet been identified and might never happen. Thus, a significant incidence of cytosolic excision could saturate all the TIM-TOM complexes of a given mitochondrion, preventing it from importing any more proteins, hydrophobic or otherwise. If this fate befell a large proportion of a cell's mitochondria, the cell itself would suffer and so a faithfully compartment-specific system is required.

One possibility might be to exploit the split inteins mentioned earlier³⁶. These have already proved to be biotechnologically attractive^{41,42}. The key to success here would be to make use of an absolutely compartment-specific feature of most cytosolically expressed mitochondrial proteins: while they are in the cytosol, they have a leader sequence that identifies them as substrates for mitochondrial import but, once they have been imported, this presequence is removed. It is likely that the C-terminal part of a split intein, which forms the N terminus of one of the progenitor proteins, would be inactivated while the mitochondrial targeting sequence was present; if so, maturation could never occur in the cytosol (Fig. 4).

Potential obstacles and suggested solutions

The clearest difficulty with this approach is that we are likely to need inteins in at least two places in at least two proteins (cytochrome b and cytochrome-c-oxidase subunit 1) in order to achieve allotropic expression of the whole mitochondrial genome. This would pose a combinatorial problem, with the risk of imported fragments being ligated to the wrong partners. It might be possible to avoid this simply by using different inteins at each site. Only one naturally occurring split intein has been discovered to date³⁶ but others have been constructed⁴¹; the fact that the N- and C-terminal intein sequences required for excision are both so long suggests that they might often be 'monogamous'.

The problem of refolding after import becomes more intimidating in this context because, if a C-terminal precursor is imported when no copies of its N-terminal partner are yet present, it must remain competent to ligate to that partner until it does arrive. In contrast to other scenarios, the C-terminal precursor is a polypeptide that is never normally present in the matrix in the absence of its N-terminal partner, so natural chaperones might be less effective. However, the ability of folded precursors to perform *trans* splicing has already been demonstrated⁴² and so this difficulty might not arise.

Finally, it is possible that the precursors – which are, after all, highly hydrophobic – would not only fold before splicing but also embed themselves in the mitochondrial membrane, thereby becoming unable to perform *trans*-splicing. One way to avoid this might be to

emulate the β subunit of *S. cerevisiae* ATP synthase, which has an anomalously long presequence. This presequence was found to act not only as a targeting peptide but also as a *cis* chaperone, preventing the mature protein from folding until after its removal, and it had the same effect when attached to other mitochondrial proteins⁴³.

Conclusion

Numerous difficulties undoubtedly remain to be surmounted before we can achieve the allotopic expression of all 13 mt-coded proteins in mammalian cells *in vitro* and then *in vivo*. However, the recent ideas and findings reviewed here give real cause for optimism that this venture will succeed in only a few years. Inteins have the potential to become the biotechnologist's most versatile new tool. The numerous means by which they might facilitate the allotopic expression of normally mt-coded proteins mark out this application as being the one in which inteins might first fulfil their enormous biotechnological potential.

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