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Structural Similarities between a Mitochondrially Encoded Polypeptide and a Family of Prokaryotic Respiratory Toxins Involved in Plasmid Maintenance Suggest a Novel Mechanism for the Evolutionary Maintenance of Mitochondrial DNA

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Summary. Subunit 8 of mitochondrial ATP synthase (A8), a mitochondrially encoded polypeptide, has no known homologue in any prokaryotic or plastid ATP synthase, suggesting that it has been recruited to its present role in the enzyme from an extraneous source. The polypeptide is poorly conserved at the primary sequence level, but shows a well-conserved hydropathy profile. The hydropathy profiles of A8 from diverse taxa were compared with those of the *hok* family of prokaryotic respiratory toxins, some of whose members are involved in plasmid maintenance, through postsegregational killing of cells that lose the plasmid at cell division. Such comparisons revealed a highly significant degree of similarity, suggesting a functional relationship. Based on these findings, it is proposed that A8 evolved from a *hok*-like protein, whose original role was the maintenance of an extrachromosomal replicon in the endosymbiont ancestor of mitochondria. An aggressive mechanism for the evolutionary maintenance of mitochondrial DNA overcomes many of the failings of traditional explanations for its retention as a separate genome.

Key words: Mitochondrial DNA — Postsegregational killing — Plasmid — Hydropathy — ATP synthase

A major outstanding puzzle in organelle biology is the evolutionary persistence of organelle DNA. Traditional explanations, based on a requirement for in situ synthesis of organelle-encoded polypeptides, or the supposed unlikelihood of gene transfer to the nucleus, have been contradicted by recent experimental studies. Genes specifying a number of mitochondrially encoded polypeptides, including very hydrophobic ones, have been successfully engineered for nuclear expression and mitochondrial import and used to complement mitochondrial mutations in the corresponding genes (Banroques et al. 1986; Nagley 1988; Nagley et al. 1988). Synthesis of these polypeptides inside mitochondria is therefore not obligatory, though the same cannot yet be asserted for all mitochondrial (mt)DNA-encoded gene products, notably the two that have been found encoded in all mtDNAs yet characterized (cytochrome oxidase subunit I and cytochrome *b*).

Transfers of mitochondrial genetic information to the nucleus over an evolutionary time-scale are well documented (Fox 1983; Jacobs et al. 1983; Fukuda et al. 1985), and have recently been demonstrated to occur at a measurable frequency in the laboratory (Thorsness and Fox 1990). In addition, the observation that a high proportion of appropriately juxtaposed random *Escherichia coli* DNA sequences is capable of supplying mitochondrial targetting information to a reporter polypeptide (Baker and Schatz 1987) suggests that mitochondrial genes acquired by nuclear transcription units during early eukaryote evolution could easily have been functional.

The alternative argument, that incompatibilities between the nuclear and organellar genetic systems have prohibited successful gene transfers to nuclear DNA also fails, because most idiosyncracies of organelle gene expression are taxon-specific. This implies that they are a consequence, not the cause, of the long period of isolated evolution. In this paper I argue that, far from being an evolutionary accident,

Table 1. Primary sequence identities between A8 polypeptides of different taxa

	Xenopus	Sea urchin	*Aspergillus*
Drosophila	15 (23)	17 (25)	10 (18)
Xenopus		20 (22)	21 (24)
Sea urchin			19 (19)

The values computed are based on N-terminal alignments without gaps. Those given in brackets are based on alignments that permit gaps, generated by the GCG BESTFIT program (Devereux et al. 1984), with gap weight set at 3.00 and gap length weight set at 0.10. The identities given are the number of identical residues in the comparison, divided by the length of the shorter sequence (including gap positions for the values in brackets), expressed as a percentage. Data used are from published mtDNA sequences of *Drosophila yakuba* (Clary and Wolstenholme 1985), *Xenopus laevis* (Roe et al. 1985), *Strongylocentrotus purpuratus* (Jacobs et al. 1988), and *Aspergillus nidulans* (Netzker et al. 1982)

the maintenance of organelle DNA may have resulted from an active mechanism to protect against its loss. Structural similarities between a mtDNA-encoded polypeptide and a protein known to be involved in an aggressive system for plasmid maintenance in *E. coli* provide evidence for such a mechanism operating during mitochondrial evolution.

Mitochondrial DNA and Plasmid Maintenance

The close resemblances between the mitochondrial transcription (Masters et al. 1987; Schinkel and Tabak 1989) and DNA replication machinery (Clayton 1982; Schinkel and Tabak 1989), and that of prokaryotic extrachromosomal elements (Kornberg 1980; Polisky 1988), suggest the possibility that the extant mitochondrial genome could have been derived from an extrachromosomal replicon of the original endosymbiont. Such a replicon would presumably have acquired a set of operons from the chromosomal genome of the endosymbiont, coding for rRNAs and tRNAs, plus certain respiratory chain components. This idea has led me to consider whether any of the multiplicity of known mechanisms for plasmid maintenance in prokaryotes (Nordström and Austin 1989) could provide a plausible model for the retention of mtDNA over evolutionary time.

One such mechanism, postsegregational killing (PSK), leads to the programmed cell death of cells that fail to inherit the plasmid. A well-characterized PSK system, specified by the *parB* locus of *E. coli* plasmid RI (Gerdes et al. 1986, 1988), codes for a suicide protein designated *hok* (host-killing). In cells that retain the plasmid, the stable mRNA for *hok* is translationally repressed by an antisense transcript (designated *sok*), which is maintained in excess by a high rate of transcription, despite its inherent instability. Plasmid loss leads to rapid decay of the *sok* RNA, permitting *hok* mRNA translation, and leading to cell death (Gerdes et al. 1988). This mechanism guards not only against plasmid loss, but also against invasion or acquisition of the plasmid by a foreign (e.g., chromosomal) transcription unit, where the balance of *hok* and *sok* RNA synthesis is likely to be disturbed by readthrough transcription.

The *hok* polypeptide acts at the cell membrane, uncoupling oxidative phosphorylation and blocking respiration (Gerdes et al. 1986). An *E. coli* chromosomal gene, *relF*, encodes a functionally similar respiratory toxin (Gerdes et al. 1986), probably required for programmed cell death under extreme starvation conditions, to permit the survival of other cells. Because a PSK mechanism for mtDNA would also be likely to act by crippling respiration, *hok/relF* provide an interesting model. The *hok* and *relF* gene products are moderately hydrophobic polypeptides of 52 and 51 amino acids, respectively. Though they share only 40% primary sequence identity, their hydropathy profiles are very similar (Gerdes et al. 1986).

Inspection of the polypeptides encoded by mtDNA in different taxa reveals that a moderately hydrophobic gene product of almost exactly this length is specified by almost all fungal, animal, and protozoan mitochondrial genomes (though it may be absent from nematode mtDNA). This polypeptide, encoded by the *aap*1 gene in yeast (Macreadie et al. 1983; Nagley 1988), usually designated ATP synthase subunit 8 (A8) in metazoa (Clary and Wolstenholme 1985; Roe et al. 1985; Fearnley and Walker 1986; Jacobs et al. 1988; Nagley 1988), is an essential, integral subunit of the F_0 portion of the proton-pumping mitochondrial ATP synthase. Its physiological function is unknown, but it has no counterpart in prokaryotic or plastid ATP synthases (Nagley 1988). The degree of sequence identity between the A8 polypeptides of organisms in different phyla (Table 1) is low, typically only about 20%. Almost half of this is contributed by the N-terminal tetrapeptide, which is usually, though not universally, MPQL. When this is discounted, the sequence identities of A8 in different organisms are hardly greater than for unrelated sequences of a similar degree of hydrophobicity. Despite the lack of conservation at the primary sequence level, A8 does show a well-conserved hydropathy profile (Clary and Wolstenholme 1985).

Hydropathy Comparisons between *hok*, *relF*, and A8

To examine whether A8 could be related to *hok*, I have compared the Kyte–Doolittle hydropathy pro-

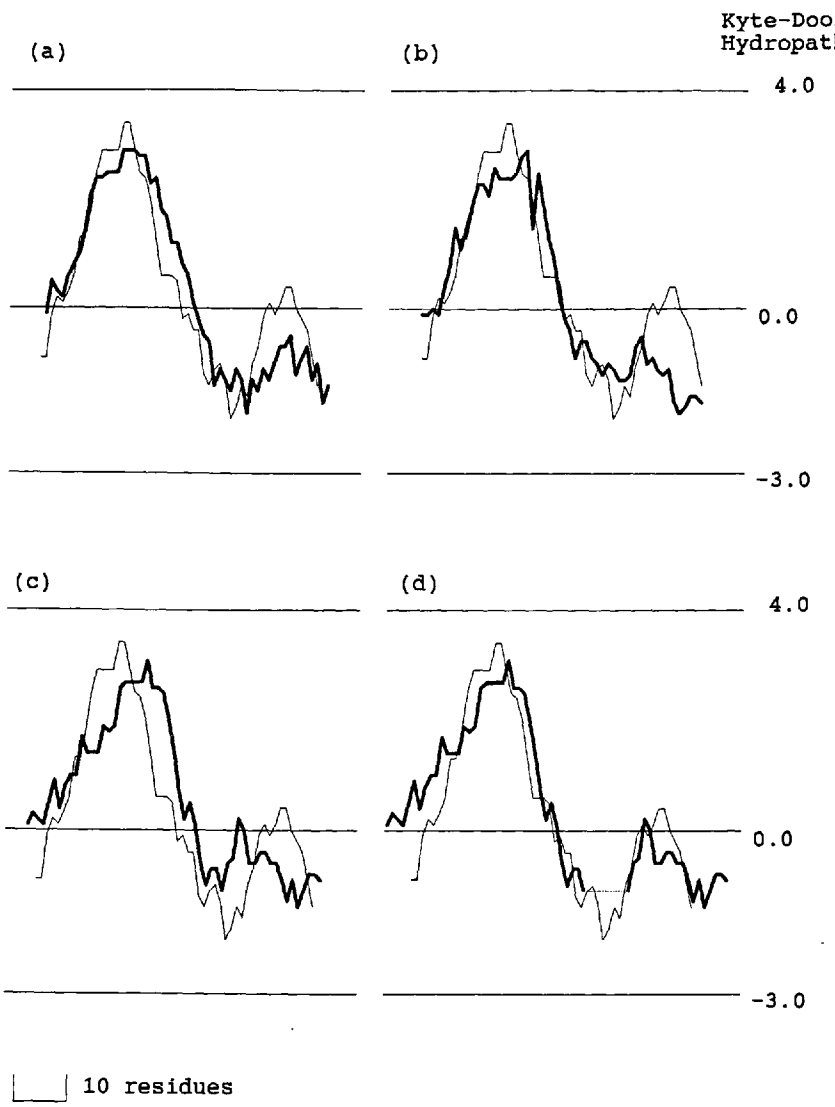

Fig. 1. Kyte–Doolittle hydropathy profiles (Kyte and Doolittle 1982) for *hok* (Gerdes et al. 1986, 1988; faint line, 11-residue window), computed using GCG program PEPTIDE-STRUCTURE (Devereux et al. 1984), compared with (bold lines) those for (**a**) *Drosophila* A8 (Clary and Wolstenholme 1985; 11-residue window, shifted by one residue to maximize alignment) and (**b**) a consensus hydropathy profile for the five sequences indicated in Table 3. Mean Kyte–Doolittle hydropathies were calculated over a 7-residue window, at each position, for the five sequences aligned individually so as to minimize the RHD value of each from *hok* (see legend to Table 2 for explanation of RHD value); (**c**) *Strongylocentrotus purpuratus* (sea urchin) A8 (Jacobs et al. 1988; 11-residue window, shifted by one residue to maximize alignment); (**d**) *Strongylocentrotus purpuratus* A8, with the N- and C-terminal halves arbitrarily interrupted (dotted line), to maximize alignment with *hok* profile. Positive values indicate hydrophobic character; negative values hydrophilic character.

files (Kyte and Doolittle 1982; Devereux et al. 1984) of mitochondrial A8 from a variety of organisms with that of *hok* (Fig. 1, Tables 2 and 3). The profiles of *hok* and *Drosophila* A8 (Fig. 1a), both 52 amino acids long (Clary and Wolstenholme 1985; Gerdes et al. 1986, 1988), are startlingly similar, despite the absence of convincing primary sequence identity (only 8–9%). The profiles of A8 from other taxa also match well to that of *hok,* as does a consensus A8 hydropathy profile built from five different sequences (Fig. 1b). The consensus profile and some individual sequences match less convincingly in their more hydrophilic C-terminal halves, but this region of A8 is quite variable between species: for example, it is extended in mammals (Fearnley and Walker 1986), but truncated and much less hydrophilic in fungi (Macreadie et al. 1983; Netzker et al. 1982). In some cases, such as the sea urchin (Jacobs et al. 1988), the N-terminal and C-terminal portions resemble *hok* very closely, but their spacing is slightly altered (see Fig. 1c and d).

Is this similarity significant? To judge this question rigorously it would be necessary to assess how often a hydropathy profile match as good as these occurs by chance in the full set of sequenced proteins. Because I am unaware of any existing computer program capable of doing this, I have sampled the databases by selecting nine confirmed or probable membrane polypeptides from phylogenetically disparate sources, having approximately the same overall length (51–58 amino acids) and hydrophobicity as *hok* (Bachmayer et al. 1968; Tanaka et al. 1975; Wu and Du 1984; Schagger et al. 1985; Power et al. 1986; Farrell and Nagley 1987; Flamm et al. 1988; Sakaguchi et al. 1988). To minimize bias, I have avoided picking more than one sequence from sets of obviously related proteins (e.g., rubredoxin/ferredoxin), and I have also avoided selecting other unidentified plasmid- or organelle-DNA-encoded polypeptides that might be related to *hok*. The sample does, however, include an unrelated subunit of ATP synthase (Farrell and Nagley 1987). I have

Table 2. Hydropathy profile differences between *hok*, *relF*, and nine other membrane polypeptides of similar overall length and hydrophobicity

Gene	RHD value from *hok*	RHD value from *relF*
A	2.41	2.53
B	2.11	2.42
C	1.92	2.38
D	2.40	2.46
E	2.11	2.07
F	2.07	2.15
G	2.19	2.52
H	2.38	2.63
I	2.11	2.27
Mean ± SE	2.189 ± 0.171	2.381 ± 0.185
relF	1.00	–

Genes used in the comparison are A, human (liver) ATP synthase proteolipid (subunit 9; Farrell and Nagley 1987); B, sea anemone anthopleurin A (sodium channel binding protein; Tanaka et al. 1975); C, yeast cytochrome *c* oxidase subunit VIIa (Power et al. 1986); D, *E. coli rts* gene (Flamm et al. 1988); E, *Peptostreptococcus* rubredoxin (nonheme iron electron acceptor protein; Bachmayer et al. 1968); F, *Chloroflexus auranticus* light-harvesting protein B-740 (bacteriochlorophyll *c* binding protein; Wechsler et al. 1985); G, human membrane glycoprotein MB-1 (Sakaguchi et al. 1988); H, bovine cytochrome *bc1* complex subunit 11 (Schagger et al. 1985); I, Chinese cobra membrane toxin D1 (Wu and Du 1984). RHD values (root-mean-square hydropathy difference) were computed from Kyte–Doolittle hydropathies (Kyte and Doolittle 1982) of the two sequences under comparison, averaged over 7-residue windows using GCG program PEPTIDESTRUCTURE (Devereux et al. 1984), and aligned from their N-termini to the end of the shorter of the two sequences (usually 51 or 52 residues). Shifting of the alignments by 1–3 residues gave no systematic alterations to RHD values. The sequences were selected by searching the (SwissProt and NBRF) protein databases for sequences of the desired length, using the GCG STRINGS program (Devereux et al. 1984). All sequences meeting the criteria described in the text were included. Mean RHD values obtained by comparing *hok* and *relF* with 60 randomly selected 52 amino acid sequences (see text) were 2.19 ± 0.29 and 2.38 ± 0.31, respectively

computed the root-mean-square hydropathy difference (RHD) per residue for the first 52 residues of each sequence compared with *hok* (see Table 2). I have also compared these nine polypeptides with *relF* in the same way. As can be seen, the RHD values for these comparisons are narrowly distributed, with means in excess of 2 units of Kyte–Doolittle hydropathy.

Because of the low informational complexity inherent in the hydropathy profiles of just nine polypeptides, and in order to detect any systematic bias introduced by confining the analysis to membrane proteins of a defined length, I have carried out a similar calculation, using a much larger set of sequences 52 amino acids in length, drawn randomly from the databases. This set of 60 sequences (30 each from the NBRF and Swissprot databases), selected by random-number-directed searching without regard for length, hydrophobicity, function, or the internal position of the 52 amino-acid block, gave RHD values in comparison with *hok* or *relF* that were almost identical with those obtained from the nine integral membrane polypeptides, but with a slightly higher variance. These RHD values appear to be an inherent property of the sequences analyzed and give a numerical indication of the degree to which their hydropathy profiles are unusual, with their close juxtaposition of extremely hydrophobic and hydrophilic regions.

Taking the RHD values from Table 2 as indicators of the degree of similarity of hydropathy profiles for *hok* or *relF* versus random polypeptides of equivalent length and overall hydrophobicity, I have examined the RHD values obtained in comparing A8 from various organisms (Table 3) with *hok*, *relF*, and with each other. All values fall at least 3 standard errors from the mean RHD for random comparisons from Table 2, based on the set of nine membrane polypeptides. If RHD values are normally distributed, these similarities are highly significant ($P < 0.01$). Moreover, the *Drosophila* A8 profile is better matched to those of *hok* or *relF* than to those of most other A8 sequences. This is a particularly striking observation, because similarity of hydropathy profile, as already indicated, is the only substantive evidence that the A8 polypeptides of different taxa are actually homologous. It may also be noted that the match of *hok* to the consensus A8 profile is only slightly worse than the match of the *hok* and *relF* profiles to each other. Overall, this approach suggests a highly significant structural similarity of A8 to *hok* and *relF*, though it must be stressed that this is a purely statistical argument, whose validity remains to be verified biochemically.

Evolutionary Significance of the A8/*hok* Similarity

The above observations do not prove that A8 and the *hok* family have a common evolutionary origin: their structural similarity could, of course, be due to convergent evolution (though this in itself does not invalidate the possibility that the proteins are functionally similar). Conversely, their structural similarity does not necessarily imply functional homology, though this is, in principle, testable.

Given these caveats, it is useful to explore the implications of a possible evolutionary and functional relationship between these proteins. On the one hand, A8 might be regarded as an evolutionary relic of the genomic maintenance system for an extrachromosomal element of the endosymbiont ancestor of mitochondria, which acquired a subset of genes for the respiratory chain, either before or after

Table 3. Hydropathy profile differences between *hok*, *relF*, and A8 from five species

Gene	RHD values from							
	dA8	bA8	xA8	aA8	sA8	conA8	*relF*	Random
hok	0.85	1.21	1.45	1.37	1.40	1.05	1.00	2.19 ± 0.17
relF	1.07	1.33	1.54	1.63	1.45	1.23	—	2.38 ± 0.19
dA8	—	1.36	1.09	1.22	1.00	0.99		
bA8		—	0.81	1.36	0.94	0.70		
xA8			—	1.53	1.40	0.89		
aA8				—	1.22	1.10		
sA8					—	0.65		
Mean ± SE						1.143 ± 0.243		

ATP synthase subunit 8 genes are denoted as follows: dA8, *Drosophila yakuba* (arthropod; Clary and Wolstenholme 1985); bA8, bovine (mammal; Fearnley and Walker 1986); xA8, *Xenopus laevis* (amphibian; Roe et al. 1985); aA8, *Aspergillus nidulans* (fungus; Netzker et al. 1982); and sA8, *Strongylocentrotus purpuratus* (echinoid; Jacobs et al. 1988). ConA8 denotes a consensus hydropathy profile for the five sequences (Kyte–Doolittle hydropathy over a 7-residue window), aligned for individual minimal RHD from *hok*, as defined in Table 1. The RHD values shown are for the best alignment of *hok* with each sequence (i.e., the one giving a minimal RHD value): this involves a shift of 1–3 residues from perfect N-terminal alignment. A clear minimum RHD value was obtained in each case, at the alignment used. The same alignments were used for comparison with *relF*, except for the *Aspergillus* sequence, where a further shift of 1 residue gave a markedly better score. The comparisons of A8 sequences with each other are all in N-terminal alignment, except for *Xenopus*/*Aspergillus*, where a 1- or 2-residue shift gave a better score. Random indicates the mean RHD value (±SE), quoted to 2 decimal places, as computed in Table 2, for the comparisons of *hok* and *relF* with unrelated sequences of similar overall length and hydrophobicity. The mean ± SE RHD value for the 10 pairwise comparisons of A8 sequences from the five species is also shown, in the conA8 column

the endosymbiotic event. Alternatively, plasmids like RI might have acquired, more recently, the preexisting property of the A8 polypeptide to interact with respiratory membranes, for destructive purposes. Two lines of argument favor the first proposition. Firstly, as already noted, an A8-related polypeptide is not found in any known prokaryotic ATP synthase. This means that the interaction of this polypeptide with proton channels would have to have been independently acquired by mitochondrial ATP synthase, plasmid RI, and the *relF* operon, from a preexisting (nondestructive) polypeptide, if it were not already manifest in the prokaryote ancestor of mitochondria. Secondly, the function of *hok* in PSK is not confined to plasmid RI: a homologous locus is found on about half of all plasmids from Gram-negative bacteria (Gerdes, personal communication), including the F plasmid (Gerdes et al. 1988). If PSK by *hok* is widespread, this suggests that it is likely to be an ancient mechanism, already present in the mitochondrial progenitor.

If A8 is a relic of an ancient PSK system for a plasmid of the mitochondrial ancestor, it would provide at least a partial explanation for the persistence of mtDNA as a separate genome. The evolutionary mechanism envisaged can be considered to comprise several steps. Initially, after endosymbiosis had occurred, a PSK system would have maintained an independent plasmid-like genome in the mitochondrion. In a second phase, this plasmid would have acquired respiratory chain genes, as well as those coding for some of the components of the mitochondrial translation apparatus. During this period, the plasmid PSK system would have acted to maintain these genes in an extrachromosomal form. In the next step, the remainder of the endosymbiont's chromosomal genome would have been lost or transferred to nuclear DNA. The PSK system would still be in place, however, and would have favored the retention of an independent mitochondrial genome, because of the cytotoxic effects of the unregulated expression of the *hok*-like gene if transferred into an active nuclear transcription unit.

The retention of other mitochondrial genes in the mtDNA replicon during this phase would not have been absolute, but would have been strongly favored by the fact that loss of any of them to a nuclear transcription unit would lead to a high probability of incorporating the *hok*-like gene into nuclear DNA by homologous recombination. This would have provided selection pressure for the eventual divergence of the nuclear and mitochondrial genetic systems. In the final step, the genetic isolation of nuclear and mtDNA would have rendered the PSK system superfluous. This would have allowed the *hok*-like gene to acquire a new function, as an essential subunit of ATP synthase.

An alternative to the above scheme, which takes account of the fact that many protistan mitochondrial genomes appear to contain significant additional coding information (Gray, personal communication), is that the respiratory chain genes may have remained chromosomal, at least in some taxa,

but that the chromosomal *relF*-like gene took on an aggressive maintenance function analogous to *hok*, to block transfer to nuclear DNA.

Rationale for the Evolutionary Retention of A8

Even if A8 originally evolved from a toxic polypeptide, by a mechanism akin to that described above, it has clearly been retained over a long period of evolution, at least in the metazoan and fungal lineages, and is now functionally indispensable for mitochondrial ATP synthase activity (Macreadie et al. 1983). How could a *hok*-related suicide protein have evolved into an essential subunit of ATP synthase?

Some possible clues are provided by the evident translational coupling between A8 and subunit 6 of ATP synthase (A6), which are translated from different, slightly overlapping reading frames of a single mRNA (Clary and Wolstenholme 1985; Roe et al. 1985; Fearnley and Walker 1986; Jacobs et al. 1988). This suggests that the synthesis of A8 and A6, which appear to bind each other, based on both genetic and biochemical evidence (Macreadie et al. 1983; Chomyn et al. 1985; Nagley 1988), must be strictly coordinated. A natural affinity of A6 for the original *hok*-like product, which later evolved into A8, could have allowed protein-level detoxification to replace antisense translational control by *sok*. This would, in turn, have allowed the A8 protein to evolve for function, so long as its synthesis was tightly regulated. It is interesting that an ectopically expressed yeast A8 gene, introduced into nuclear DNA, did not restore full wild-type ATP synthase activity to an *aap*1 mutant, possibly due to unregulated expression of A8 resulting in a partial uncoupling of oxidative phosphorylation (Nagley, personal communication).

The data and hypothesis presented here raise important questions regarding the physiological role and regulation of present-day A8. Is there a uniform stoichiometry, for example, between A8 and other subunits of ATP synthase in different metazoan tissues? What is the mechanism of translational coupling between A8 and A6, and what are the consequences of over-expression of either subunit? Is there any evidence for interactions between A8 and other respiratory centers? Most importantly, can direct evidence be obtained of a functional homology between A8 and *hok*, by expressing A8 or chimeric A8/*hok* polypeptides in bacteria? To answer these questions, it will be necessary to carry out a detailed analysis of the properties of the A8 polypeptide from metazoans, by engineering the A8 gene for expression outside of mitochondria.

Plastid DNAs are clearly different from mtDNAs in that their gene content is much higher, and their gene expression machinery of a more conventionally prokaryotic type. Plastid ATP synthases are also more akin to those of bacteria and lack subunit 8. It is tempting to speculate that the evolutionary maintenance of plastid DNA may also have been achieved by an aggressive mechanism, but a quite different one from the PSK system hypothesized here for mtDNA. Examination of plasmid stability mechanisms in cyanobacterial replicons may provide instructive clues to this additional puzzle.

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