Cryptic Organelles in Parasitic Protists and Fungi

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ABSTRACT

A number of parasitic protists and fungi have adopted extremely specialised characteristics of morphology, biochemistry, and molecular biology, sometimes making it difficult to discern their evolutionary origins. One aspect of several parasitic groups that reflects this is their metabolic organelles, mitochondria and plastids. These organelles are derived from endosymbiosis with an alpha-proteobacterium and a cyanobacterium respectively, and are home to a variety of core metabolic processes. As parasites adapted, new demands, or perhaps a relaxation of demands, frequently led to significant changes in these organelles. At the extreme, the organelles are degenerated and transformed beyond recognition, and are referred to as "cryptic". Generally, there is no prior cytological evidence for a cryptic organelle, and its presence is only discovered through phylogenetic analysis of molecular relicts followed by their localisation to organelle-like structures. Since the organelles are derived from eubacteria, the genes for proteins and RNAs associated with them are generally easily recognisable, and since the metabolic activities retained in these organelles are prokaryotic, or at least very unusual, they often serve as an important target for therapeutics. Cryptic mitochondria are now known in several protist and fungal parasites. In some cases (e.g., *Trichomonas*), well characterised but evolutionarily enigmatic organelles called hydrogenosomes were shown to be derived from mitochondria. In other cases (e.g., *Entamoeba* and microsporidia), "amitochondriate" parasites have been shown to harbour a previously undetected mitochondrial organelle. Typically, little is known about the functions of these newly discovered organelles, but recent progress in several groups has revealed a number of potential functions. Cryptic plastids have now been found in a small number of parasites that were not previously suspected to have algal ancestors. One recent case is the discovery that helicosporidian parasites are really highly adapted green alga, but the most spectacular case is the discovery of a plastid in the Apicomplexa. Apicomplexa are very well-studied parasites that include the malaria parasite, *Plasmodium*, so the discovery of a cryptic plastid in Apicomplexa came as quite a surprise. The apicomplexan plastid is now very well characterised and has been shown to function in the biosynthesis of fatty acids, isopentenyl diphosphate and heme, activities also found in photosynthetic plastids.

1. INTRODUCTION

The fundamental difference between prokaryotes and eukaryotes is the compartmentalisation of the cell. The primary defining feature of

eukaryotic cells is the enclosure of the bulk of their DNA within the nucleus, and eukaryotes are further characterised by the compartmentalisation of numerous cellular processes within membrane-bounded structures, or organelles. These include the Golgi apparatus, endoplasmic reticulum, peroxisomes, lysosomes, endosomes, mitochondria and plastids. The origins of these structures have been the subject of considerable debate and speculation for which there are two generally contrasting possibilities: an autogenous origin (i.e., derived from within the cell) versus an endosymbiotic origin (i.e., from a merger of two cells). The endomembrane system – composed of the endoplasmic reticulum, Golgi apparatus, endosomes, lysosomes, and the nucleus – is a continuous system and is widely held to have originated autogenously by invagination of vesicles of the plasma membrane. The nucleus has, from time to time, been suggested to be derived from an endosymbiotic bacterium based on a variety of rationales (e.g., Lake and Rivera, 1994; Lopez-Garcia and Moreira, 1999). However, the fact that the nucleus is continuous with the endomembrane system cannot easily be explained by phagocytosis of a bacterial cell, and endosymbiotic models for nuclear origins often mistakenly claim it to be a double membrane-bounded organelle, but it is really a single membrane folded on itself. Therefore, the more widely held view is that the nucleus resulted from the infolding of a primitive endoplasmic reticulum (Cavalier-Smith, 1991b). Similarly, the peroxisome has been proposed to be derived from a bacterial endosymbiont, partly based on the observation that they appear to grow and divide by fission (Lazarow and Fujiki, 1985). However, peroxisomes have no genome with which to trace a possible exogenous origin and, furthermore, it has been shown that their replication is dependent on the endoplasmic reticulum – suggesting a possible relationship to the endomembrane system (Titorenko *et al*., 1997). The current view thus appears to be that peroxisomes are also autogenously derived organelles (Martin, 1999; Cavalier-Smith, 2002). In contrast, it is now abundantly clear, based on a variety of evidence from morphology, biochemistry, and molecular phylogeny, that mitochondria and plastids are derived exogenously from endosymbiosis events involving eubacteria.

Mitochondria and plastids are structurally very distinctive and are almost universally recognisable by their characteristic features. The mitochondrion is often considered one of the defining structures of the eukaryotic cell and is thought to be virtually ubiquitous in this group. The plastid is similarly considered a defining feature of plants and algae. However, striking variations on the basic structure of these organelles do exist and, not surprisingly, this is most often seen when organisms make extreme adaptive changes that create new demands on the metabolic pathways that these

organelles harbour. One common circumstance that appears to drive such change is the adaptation to a parasitic way of life.

In many groups of parasitic eukaryotes, the mitochondria and plastids have been substantially altered in morphology and biochemistry. Whilst these peculiar organelles are interesting from an evolutionary perspective, a thorough understanding of them also offers practical gains: they frequently contain unique biochemical pathways not found in other eukaryotes, and these differences provide new potential drug targets (McFadden and Roos, 1999). At its most extreme, parasitism leads to such a significant alteration in the organelle's ultrastructural features and biochemical pathways that they are no longer recognisable, and become "cryptic" organelles. Fortunately, in the case of mitochondria and plastids, their relict bacterial genome or other molecular clues can be used to find these organelles and determine their evolutionary origin, even when all other archetypal features have been lost. Here we review a number of instances in which mitochondria and plastids in parasitic protists and fungi have been modified to the extent that they are difficult to recognise. In each case, reconstructing molecular phylogenies has been instrumental in identifying the evolutionary origin of these organelles, in determining their function by identifying which enzymes and biochemical pathways are localised to the organelle, or even in hinting at the very existence of the organelles themselves.

2. THE ORIGIN OF MITOCHONDRIA AND PLASTIDS BY ENDOSYMBIOSIS

2.1. Mitochondria

As previously stated – mitochondria are unquestionably derived from endosymbiosis. Mitochondria were first recognised under the light microscope in the 1850s and approximately 100 years later they were recognised as housing the components of fatty acid oxidation, respiration, and oxidative phosphorylation, leading to the label, the 'powerhouse of the cell' (Kennedy and Lehninger, 1949). Fifty years on, mitochondria have been shown to have far wider roles than solely ATP production, and it is now known that their other essential functions include iron sulphur cluster assembly and export, and programmed cell death, amongst others (Kroemer *et al*., 1995; Lill *et al*., 1999). Surprisingly, as early as 1890 mitochondria were suggested to be autonomous structures derived from symbiotic bacteria (Altmann, 1890). This hypothesis was resurrected several times over the next century, but was not widely accepted until its popularisation during the 70s and 80s.

The debate over the endosymbiotic origin of mitochondria cannot be separated from the parallel debate over the origins of plastids, although the evidence for the endosymbiotic origin of mitochondria lagged behind the much clearer case for plastids (see below). By 1982 a long list of characteristics suggested very strongly that plastids had an endosymbiotic origin (Gray and Doolittle, 1982). These included the sensitivity of chloroplast protein synthesis to anti-bacterial antibiotics, biochemical and ultrastructural similarities to cyanobacteria, ribosomes with a eubacterial sedimentation coefficient, and, most importantly, the presence of a small genome that retained several eubacterial features. Several such characteristics were also found for mitochondria, but the endosymbiotic origin of the mitochondrion from a prokaryotic cell was unequivocally established by the discovery and phylogenetic analysis of its genome. The mitochondrial genome is generally, but not always, a single circular chromosome, and is highly reduced compared with the genomes of its autonomous eubacterial relatives (Gray and Spencer, 1996; Gray *et al*., 2001) (Table 1). Nevertheless, it contains a variety of genes now known to be eubacterial in nature. This was first revealed through phylogenetic analysis based on cytochrome C protein sequences and small subunit ribosomal RNA (SSU rRNA) (Schwartz and Dayhoff, 1978; Yang *et al*., 1985). SSU rRNA phylogeny clearly placed mitochondria closer to prokaryotes than to eukaryotes, and

Table 1 Size and characteristics for organellar genomes.

Organism	Organelle	Genome
Homo (animal)	mitochondrion	16.5 kbp, circular
Saccharomyces (fungus)	mitochondrion	85.8 kbp, circular
Arabidopsis (plant)	mitochondrion	367 kbp, linear (?)
Porphyra (red alga)	mitochondrion	36.8 kbp, circular
Plasmodium (apicomplexan)	mitochondrion	6 kbp, linear
Reclinomonas (jakobid)	mitochondrion	69 kbp, circular
Entamoeba (archamoeba)	mitosome	Uncertain
Encephalitozoon (microsporidian)	mitosome	None
Trichomonas (parabasalian)	hydrogenosome	None
Neocallimastix (chytrid fungus)	hydrogenosome	None
Nycotherus (*ciliate*)	hydrogenosome	present, but uncharacterised
Arabidopsis (plant)	plastid	154.5 kbp, circular
Epifagus (parasitic plant)	plastid	70 kbp, circular
Porphyra (red alga)	plastid	191 kbp, circular
Plasmodium (apicomplexan)	plastid (apicoplast)	35 kbp, circular
Prototheca (green alga)	plastid	54 kbp, circular
Helicosporidium (green alga)	plastid	present, but uncharacterised

also allowed the identification of the closest ancestral lineage of the mitochondrion, the alpha subdivision of the proteobacteria (Yang *et al.*, 1985). Interestingly, this ancestor was previously proposed on the basis of biochemical data (Whatley, 1981), and has now been widely supported by the analysis of key genomic sequences (Kurland and Andersson, 2000).

The origin of the mitochondrion is thought to have occurred something like the process shown in Figure 1. In this simplified scheme, an early eukaryote, or perhaps a protoeukaryote (it is not certain that this organism possessed a nucleus at this point) engulfed an alpha-proteobacterial cell. This suggests that the host must have at least developed a system for phagocytosis, and therefore the eukaryotic cytoskeleton was likely a key innovation that allowed the evolution of organelles by endosymbiosis

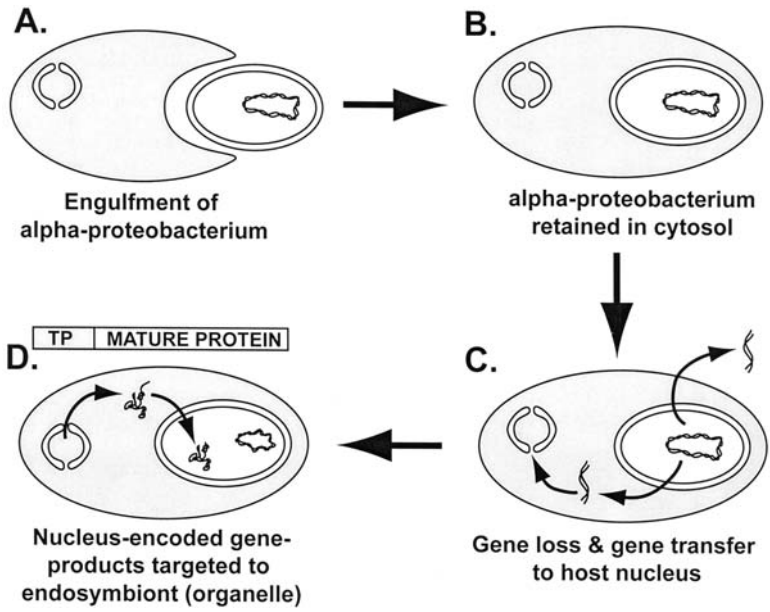

Figure 1 Schematic of the endosymbiotic origin of mitochondria. (A) A heterotrophic eukaryote (or some proto-eukaryote) engulfed an alpha-proteobacterium and (B) retained it within the cytoplasm, losing the membrane of the phagocytic vacuole. As the endosymbiotic relationship progressed (C), the endosymbiont lost genes no longer necessary and transferred others to the nuclear genome of the host. A system was established to target proteins to the organelle using an N-terminal leader sequence called a transit peptide (TP), so that protein products of transferred genes are localised to the organelle. A schematic of one such protein is shown in (D). All canonical mitochondria possess a small relict genome, but several mitochondrion-derived organelles have lost the genome altogether.

(Stanier, 1970). In any case, rather than digesting this alpha-proteobacterium for nutrients, it was retained in the cytosol for reasons that are not presently established. Various theories have been put forward to explain this association, which have been discussed elsewhere (e.g., Martin and Müller, 1998; Gupta, 1999; Lopez-Garcia and Moreira, 1999; Kurland and Andersson, 2000). The endosymbiont and host then became increasingly mutually dependent and integrated. Part of this process was a large-scale reduction of the endosymbiont at many levels. Many structural and biochemical characteristics of the bacterium were discarded, including the peptidoglycan wall, flagella, as well as most of its biochemical versatility. At the molecular level, this reduction meant a significant decrease in size of the endosymbiont genome (Gray *et al*., 1999; Kurland and Andersson, 2000). Much of this reduction reflects the loss of a large number of genes as their products became unnecessary, but the mitochondrial genome is still far too small to encode all of the genes required for those structural, biochemical, and housekeeping functions retained by the organelle. Most of these genes were transferred to the nucleus of the host cell, and their protein products are translated by cytosolic ribosomes and post-translationally targeted to the mitochondrion. This post-translational targeting system is generally mediated by distinctive N-terminal leader sequences called transit peptides on proteins bound for the mitochondrion. These are recognised by protein complexes in the outer and inner membranes of the mitochondrial envelope (Pfanner and Geissler, 2001). Many of the proteins in these membrane complexes are referred to as TIMs and TOMs (*T*ranslocation *I*nner *M*itochondrial membrane and *T*ranslocation *O*uter *M*itochondrial membrane, respectively), and these are assisted by several molecular chaperones that refold the proteins as they cross the membranes.

The near-ubiquitous distribution of mitochondria across the eukaryotic tree implies that the endosymbiotic origin of mitochondria occurred at an early stage of eukaryotic evolution, earlier than the endosymbiosis that gave rise to plastids. This suggests that eukaryotes that appear to lack mitochondria may represent extremely ancient eukaryotic lineages that diverged from a pre-mitochondrial ancestor. This idea was formalised in the Archezoa hypothesis, in which organisms that were thought to be primitively amitochondriate were placed within a paraphyletic group at the base of the eukaryotic tree (Cavalier-Smith, 1983, 1987). This group included the microsporidia (e.g., *Encephalitozoon*), the parabasalids (e.g., *Trichomonas*), the diplomonads (e.g., *Giardia*), retortomonads and oxymonads, and the Archamoebae (e.g., *Entamoeba*). Phylogenetic analysis of the first molecular data from these organisms appeared to provide stunning support for this theory: the SSU rRNA from microsporidia, Parabasalia, and diplomonads all clustered at the base of the eukaryotes

with strong support (Vossbrinck *et al*., 1987; Sogin *et al*., 1989; Sogin, 1989). Initial analyses of protein-coding genes also supported this view (Hashimoto *et al*., 1994; Shirakura *et al*., 1994; Kamaishi *et al*., 1996a,b) and the phylogenetic debate was centred largely on which archezoan was the most primitive of all (Leipe *et al*., 1993). In the end, phylogenetic analyses of other genes demonstrated that none of the Archezoa were primitively amitochondriate (discussed below), and there is currently no direct evidence that any primitively amitochondriate eukaryote exists today.

2.2. Plastids

Plastids are the cellular compartments of plants and algae where photosynthesis takes place. The term plastid is a general term for any photosynthetic organelle and all their non-photosynthetic derivatives. Other terms refer to specific functional or phylogenetic subsets of plastids. For example, chloroplasts are photosynthetic plastids of plants, green algae and their derivatives (e.g., those of *Chlorarachnion* and *Euglena* – see below), while cyanelles are the plastids of glaucophyte algae. Some examples of functionally defined subsets of plastids include a variety of non-photosynthetic organelles such as leucoplasts, amyloplasts, and eliaoplasts.

As with mitochondria, there is now an abundance of evidence from morphology, biochemistry, cell biology, and molecular sequences supporting an endosymbiotic origin of plastids from a cyanobacterium (McFadden, 2001). Indeed, the first musings of a possible endosymbiotic origin of any organelle came from observing plastids under the light microscope (Schimper, 1883; Mereschkowsky, 1905). This idea received little attention for some time, but was rekindled in the mid-20th century for a number of the same reasons discussed for mitochondria, most importantly the discovery that the organelle contained DNA (Stocking and Gifford, 1959). Plastid genomes are, like most mitochondrial genomes, composed of a single circular chromosome that is highly reduced, but generally not so reduced as mitochondrial genomes (see Table 1).

It has been clear for some time that all plastids are derived from cyanobacteria. In addition to a strong relationship between nearly all plastid genes and their cyanobacterial homologues, plastids and cyanobacteria share a large number of extremely detailed and unique biochemical characteristics relating to photosynthesis. There are four bacterial groups where photosynthesis takes place, but the cyanobacteria and plastids are unique in possessing both photosystems I and II, and by generating molecular oxygen by splitting water molecules (Blankenship, 1994). While the cyanobacterial origin of plastids is not disputed, the huge diversity of

plastid types, together with the even greater diversity of host cells in which plastids are found led to a great deal of controversy over the origin of plastids and their subsequent evolutionary history. Part of this controversy resulted from the fact that plastids are scattered all over the tree of eukaryotes: a number of algae are clearly more closely related to non-photosynthetic protists than to other groups of algae (e.g., the photosynthetic *Euglena* and the non-photosynthetic *Trypanosoma*). Such a distribution is consistent with multiple independent plastid origins, but the truth turned out to be much stranger. Unlike mitochondria, where the eubacterium was engulfed, retained and inherited faithfully thereafter, plastids have continued to spread from eukaryote-to-eukaryote by a process called secondary endosymbiosis (Archibald and Keeling, 2002). In this case (Figure 2), primary endosymbiosis refers to the engulfment and retention of the cyanobacterium by a eukaryote, which gave rise to so-called 'primary plastids' found in glaucocystophytes, red algae, green algae, and plants (which evolved from green algae). While this is a large and diverse collection of algae, it is only a fraction of the total diversity of photosynthetic eukaryotes. Most algal groups acquired photosynthesis by engulfing one of these primary algae (either a red or a green, never a glaucocystophyte), and converting this primary alga into an organelle by a process of reduction and gene transfer similar to what is seen in primary endosymbiosis. In most cases, this process resulted in a plastid distinguished only by extra membranes corresponding to the phagosomal membrane of the secondary host and the plasma membrane of the primary alga (dinoflagellates and euglenids have only three membranes and it is thought that the primary algal plasma membrane has been lost in these plastids). In two cases, however, a relict algal nucleus with a miniaturised genome also persisted between the second and third membranes (McFadden *et al*., 1997).

An understanding of secondary endosymbiosis helps explain plastid diversity and shows quite clearly how plastids can be distributed across the tree of eukaryotes, but it leaves a few questions unsolved, and raises a few new ones. There has long been a debate over whether plastids ultimately trace back to a single primary endosymbiosis or whether they arose by several independent endosymbiotic events involving different cyanobacteria. Currently, two lines of evidence very strongly favour the former hypothesis. First, in practically all phylogenies that have been constructed, the plastids form a single clade within the cyanobacteria. Second, certain characteristics of the plastid proteomes and genomes are unique to plastids. Such features include the rRNA inverted repeat, the so-called plastid superoperon, and three-helix light-harvesting antennae proteins, none of which are found in cyanobacteria (Palmer, 2003). Between these characteristics and the phylogenetic trees, there is no good evidence for multiple origins of plastids

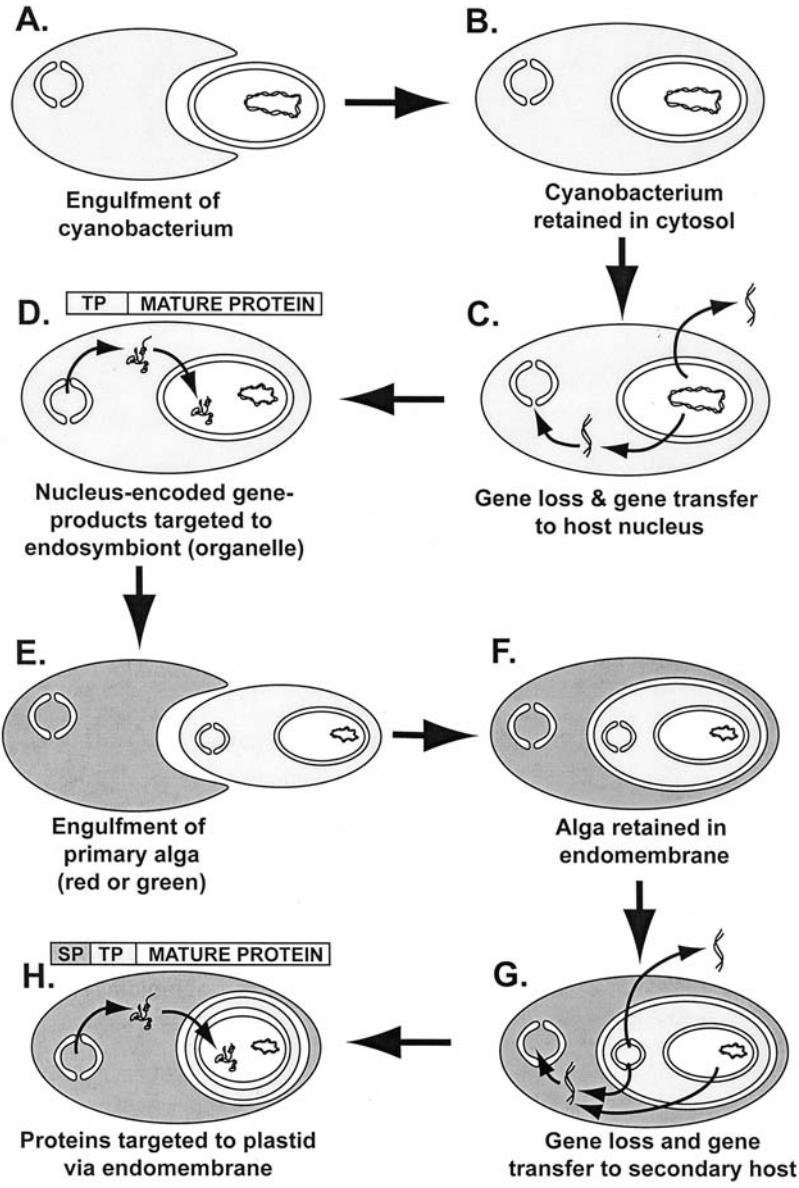

Figure 2 Schematic of the endosymbiotic origins and spread of plastids. (**A–D**) In events similar to the origin of mitochondria (Figure 1), a heterotrophic eukaryote engulfed and retained a cyanobacterium. The endosymbiont and host were integrated in a process involving many gene losses and gene transfers, and the

(see Stiller *et al*., 2003), although one interesting potential exception is a little known amoeba called *Paulinella chromatophora*, which may have independently acquired its plastid (Kies, 1974). In addition, the discovery that plastids could be transferred from one lineage to another has led to considerable debate as to how many times this has happened and what kinds of cells were involved. Current evidence favours three secondary endosymbiotic events: two involving green algae and one involving a red alga (see Figure 3), the latter being discussed in more detail below.

Secondary endosymbiosis also has profound implications for protein trafficking. Primary plastids, like mitochondria, have transferred the bulk of their genetic material to the host nucleus, and the protein products of these genes are post-translationally targeted to the plastid. There is some variation in the method of translocation, but typically a transit peptide is recognised by membrane proteins called TICs and TOCs (*T*ranslocation *I*nner *C*hloroplast membrane and *T*ranslocation *O*uter *C*hloroplast membrane, respectively), and the protein is moved across both membranes using these protein complexes and several molecular chaperones, much like protein translocation in mitochondria (Cline and Henry, 1996; Hiltbrunner *et al*., 2001). Secondary plastids, however, are not actually in the cytoplasm of the secondary host, but are in the endomembrane system (the outermost membrane is derived from the phagosome of the secondary host). Proteins expressed in the cytosol of these cells are not exposed to the TOCs, and could not be targeted with a transit peptide alone. All algae with secondary plastids have overcome this problem in the same general fashion: plastid-targeted proteins encode complex, bipartite leaders with a signal peptide followed by a transit peptide (some have additional information to move the

establishment of a protein-targeting apparatus similar to that of mitochondria. These plastids (**D**) are called primary plastids and are found in glaucocystophytes, red algae, green algae, and land plants. Proteins are targeted to primary plastids using a system much like that of mitochondria, and these proteins have N-terminal transit peptides as shown in (**D**). In secondary endosymbiosis (**E**–**F**), a second heterotrophic eukaryote then engulfs and retains one of these primary plastid-containing algae, and the eukaryotic algae is itself reduced to an organelle. This involves the transfer of many genes from the algal nucleus to the new host nucleus (**G**), and the establishment of a more complex protein-targeting system (**H**) to direct the protein products of these genes to the plastid via the endomembrane system using a signal peptide (SP), as well as the original transit peptide (TP). A schematic of a protein with such a complex leader is shown in (**H**). Secondary plastids are found in euglenids, chlorarachniophytes, cryptomonads, heterokonts, haptophytes, dinoflagellates, and apicomplexans. All plastids contain a small genome, and in two cases, the secondary endosymbiotic algal nucleus has been retained in a much degenerated state.

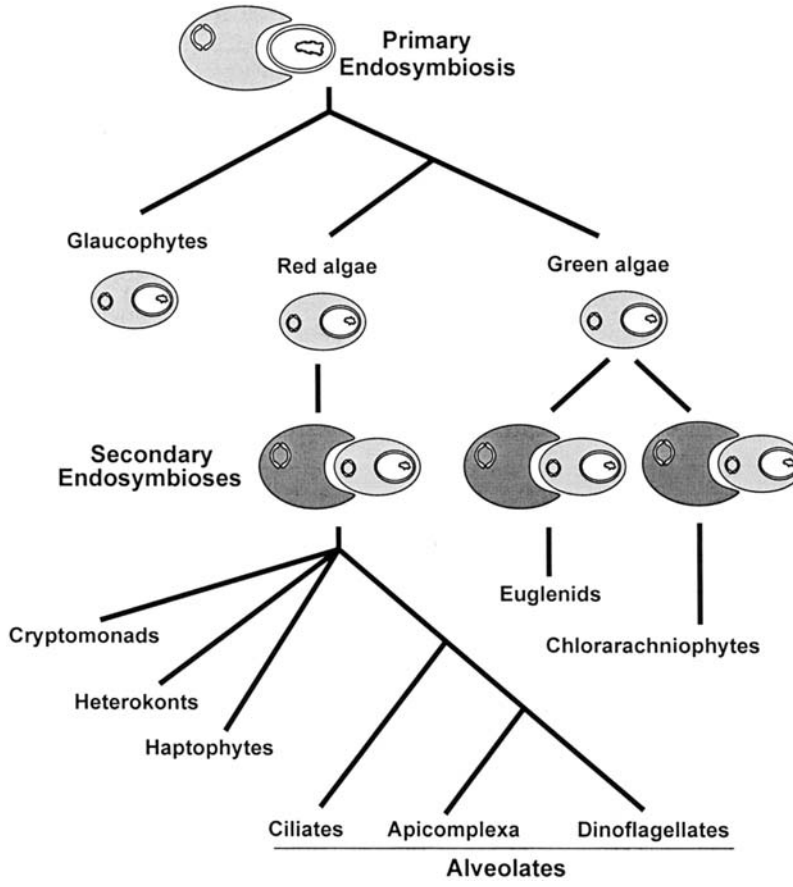

Figure 3 Schematic of the evolutionary history of plastids in eukaryotes. Primary endosymbiosis involving a cyanobacterium (Top) led to glaucocystophytes, red algae, and green algae (plants are derived from green algae). Three secondary endosymbiosis (Bottom) involving two independent green algae (Right) and one red alga (Left) resulted in the remaining plastid-containing eukaryotes. The single origin of all red algal secondary plastids has been demonstrated phylogenetically, and has important implications for the plastids of several parasitic protists.

protein to the thylakoid within the plastid). The signal peptide directs the partially translated protein and ribosome to the rough endoplasmic reticulum, where the protein is inserted into the endomembrane system co-translationally, thus crossing the outermost membrane (McFadden, 1999). The transit peptide ensures that the protein can pass through the two

inner, cyanobacterium-derived membranes, but how the protein crosses the membrane derived from the primary algal plasma membrane is unknown. This added complexity in targeting is a signature for nuclear genes encoding proteins targeted to secondary plastids.

3. CRYPTIC ORGANELLES AND HOW TO FIND THEM

In most eukaryotes, mitochondria appear normal and function in the expected way. Similarly, virtually all algae are photosynthetic and their plastids are easily distinguishable and function normally. Even in cases where an alga has lost photosynthesis secondarily, the plastid is generally easily recognisable (Sepsenwol, 1973; Siu *et al*., 1976; Sekiguchi *et al*., 2002). In certain cases, however, the evolutionary specialisation of a group has been more extreme, and one reflection of this can be a reduction or transfiguration of the organelle beyond recognition. Very often these cytologically unrecognisable, or cryptic organelles were overlooked, and the organism was considered to be amitochondriate or aplastidial until closer inspection revealed an organelle drastically different in morphology and biochemistry than its antecedents.

One mode of life that often precipitates extreme evolutionary adaptation is parasitism. Many parasites are otherwise quite normal, but certain groups have evolved extremely sophisticated modes of parasitism, increasingly adapting to infect other organisms or other cells, typically at the expense of autonomy. Often, but not always, the most highly adapted parasites are extremely dependent upon their host. As a result they have discarded a number of biochemical capabilities, significantly altered their cellular morphology, and re-tooled their metabolism to take advantage of their new niche. In some cases it is difficult to know whether the adaptation to parasitism drove these changes, or if these cells were strange free-living organisms that later adapted to parasitising other cells. In either case, parasites offer an abundance of variation in many of the core components of the eukaryotic cell, not the least of which are organelles.

In the case of the mitochondrion, the otherwise ubiquitous nature of the organelle has always drawn immediate attention to any eukaryote that lacks it (Müller, 1992; Embley and Hirt, 1998; Sogin and Silberman, 1998; Lang *et al*., 1999; Keeling and Fast, 2002). Moreover, many amitochondriates are important parasites, in part because one of the abundant anoxic niches available to amitochondriates is inside other cells or organisms. Accordingly, there has been considerable effort put into actively looking for mitochondria in many putative amitochondriate parasites. Electron

microscopy and biochemical analysis have both been applied to many of the amitochondriate parasites, but these organisms are highly adapted to living in anoxic or microaerophilic environments, so it is perhaps (in retrospect) not surprising that mitochondria were often not readily apparent by either means. Mitochondrial DNA was also sought, but in the vast majority of cases was not found (see below). In a special relative of mitochondria, hydrogenosomes, the actual organelle was discovered and well characterised cytologically and biochemically (Lindmark and Müller, 1973), but these are so unlike mitochondria in form and function that their relationship to mitochondria was not obvious at the time (Müller, 1973, 1993, 1997).

Ultimately, the secrets of cryptic mitochondria were given up by the nuclear genome of the host. Recall that the genomes of normal mitochondria are highly reduced: those genes that have been retained in most mitochondrial genomes encode proteins involved in gene expression or respiration, and these genes (and therefore the genome itself) would probably be lost relatively quickly if the organism adapted to an anoxic environment. However, most mitochondrial genes were transferred to the host nucleus and the proteins are post-translationally targeted to the organelle (Figure 1). It was reasoned that even if the organelle was significantly altered (and perhaps even if completely lost) some of these mitochondrion-derived proteins would retain an indispensable activity and the genes would remain in the nucleus (Clark and Roger, 1995). The genes that were most widely chosen to search for clues of a cryptic mitochondrion were molecular chaperones (heat shock proteins) that are part of the protein import system of all mitochondria. In most cases, genes encoding the 60 kDa chaperonin (Cpn60) or the 70 kDa class of heat shock protein (HSP70) were the critical markers for missing mitochondria, although HSP10, pyridine nucleotide transhydrogenase, and pyridoxal-5′-phosphate-dependent cysteine desulfurase also played important roles in the search (Clark and Roger, 1995; Bui *et al*., 1996; Germot *et al*., 1996, 1997; Horner *et al*., 1996; Hirt *et al*., 1997; Peyretaillade *et al*., 1998; Roger *et al*., 1998; Morrison *et al*., 2001; Tachezy *et al*., 2001; Williams *et al*., 2002). Chaperones involved in protein import were logical choices because their activity is critical, no matter what the function of the organelle may be, and because they are typically highly conserved and well-sampled genes that are amenable to molecular phylogenies. Finding these genes and demonstrating that they are phylogenetically related to other mitochondrial homologues, provides compelling evidence that the organisms in which they are found are descended from mitochondriate ancestors, but it does not necessarily prove that they still harbour the organelle, despite the fact that in many cases the proteins were found to encode N-terminal leaders that resembled transit peptides

(Clark and Roger, 1995; Bui *et al*., 1996). The most important evidence for the presence of the organelle itself comes from using the proteins as markers for the organelle by immunocytochemistry. By raising antibodies against the newly characterised mitochondrial chaperones it is possible to determine where in the cell these proteins accumulate, and to find the organelle (e.g., Mai *et al*., 1999; Tovar *et al*., 1999; Williams *et al*., 2002).

The situation with plastids is much different than that of mitochondria, since plastids are restricted in distribution by virtue of their relatively late introduction into eukaryotes (Archibald and Keeling, 2002). Accordingly, a plastid-lacking eukaryote does not attract much attention based on that character alone unless it is demonstrated to have evolved from photosynthetic ancestors. Therefore, cryptic plastids have been discovered by two different routes: an unusual organism is found to have evolved from an alga and the plastid is then sought directly, or a cryptic plastid is discovered quite by chance in an organism where it is not expected to exist.

Also, in contrast to the situation with mitochondria, outright loss of the plastid genome seems to be extremely rare, even in the most highly derived plastids (although see Nickrent *et al*., 1997b). Why this is the case is not clear, but in general, plastid genomes are less reduced than those of mitochondria and encode genes relating to a greater diversity of metabolic processes than mitochondrial genomes (Wolfe *et al*., 1992; Nickrent *et al*., 1998; Palmer and Delwiche, 1998; Gray, 1999). Most organelle genes encode components of the gene expression machinery, so they are not intrinsically essential (i.e., if the genome is lost, they are no longer essential). In the mitochondria, most other genes are for proteins involved in respiration, so if respiration were lost, the genome would also presumably be lost. In contrast, plastid genomes generally encode proteins involved in a number of processes other than transcription, translation, and photosynthesis, which may make it more difficult for a plastid genome to be eliminated. The retention of a genome also has important practical implications. It means that the cell retains many more direct and obvious molecular clues with which to investigate the presence of a plastid, and the localisation of the organelle can be much more straightforward technically, since the genome or its rRNA gene products can be the target for localisation (which requires DNA probes) rather than proteins encoded by nuclear genes (which requires antibodies).

Even when a cryptic organelle is detected and identified, however, the mystery is only partly solved, since the major question is, ‘‘what is it doing in the cell?’’ Below, we will outline the history of the discovery of several cryptic mitochondria and plastids in a variety of important parasitic protists and fungi, and we will bring together the (sometimes scant) evidence for what role these unusual organelles play in their hosts.

4. CASE HISTORIES – MITOCHONDRIA

4.1. Hydrogenosomes

Hydrogenosomes were first described biochemically in 1973 in the parabasalian parasite, *Tritrichomonas foetus* (Lindmark and Müller, 1973). Although the structure corresponding to this organelle had been previously recognised in other trichomonad Parabasalia, it was assumed to be an aberrant peroxisome or mitochondrion. However, Lindmark and Müller recognised that the organelle housed a unique biochemistry, in which molecular hydrogen was produced through the oxidation of pyruvate, distinguishing it from both peroxisomes and mitochondria. Hydrogenosomes have subsequently been discovered to be common to all parabasalids, and have also been found in a variety of other phylogenetically distantly related organisms, including certain anaerobic ciliates and chytrid fungi. Ultrastructural data also suggest that hydrogenosomes may be present in the amoeboflagellate heterolobosean *Psalteriomonas lanterna* (Broers *et al*., 1990), the euglenozoan *Postgaardi mariagerensis* (Simpson *et al*., 1997), *Trimastix pyriformis* (O'Kelly *et al*., 1999), and the enigmatic flagellate *Carpediemonas membranifera* (Simpson and Patterson, 1999), which is closely related to diplomonads and retortamonads (Simpson *et al*., 2002). However, there is currently little biochemical data about the activities of these organelles.

All hydrogenosome-bearing organisms are linked by the ecological trait of living in anaerobic or microaerobic environments and, where it has been examined, the organelle produces ATP using hydrogen rather than oxygen as the terminal electron acceptor (Müller, 1993). The hydrogenosomes from each of these organisms contain comparable, though not identical biochemistry (Hackstein *et al*., 1999). The long history of hydrogenosome research indicates that these are not, strictly speaking, "cryptic" organelles, however, their evolutionary origins and the relationships between hydrogenosomes in various groups have been a source of much debate.

4.1.1. Ciliate hydrogenosomes

While most ciliates are aerobic and contain typical mitochondria, a small number of ciliates have been found to contain hydrogenosomes (Fenchel and Finlay, 1995). These anaerobes are typically found in anoxic freshwater and marine sediments or in the rumen or caecum, where they exist in complex associations with their animal hosts and other anaerobic protists and fungi. The hydrogenosomes of these ciliates have not been studied in detail, but

their metabolism has been worked out to some extent (Müller, 1993), and their hydrogenosomes have received ever greater attention in recent years.

It is evident that the hydrogenosome-containing ciliates and chytrid fungi both evolved from mitochondriate ancestors, as most ciliates and fungi are mitochondriate and the hydrogenosome-containing members are phylogenetically positioned well within these otherwise mitochondriate groups. Given that the hydrogenosomal ciliates and fungi all lack mitochondria, it has generally been considered most parsimonious that the hydrogenosomes in these groups evolved from pre-existing mitochondria (Embley and Hirt, 1998). This is especially clear in ciliates, since their hydrogenosomes are bound by a double membrane and ultrastructurally similar to the mitochondrion (Embley *et al*., 1997). Molecular phylogenies based on SSU rRNA have shown that hydrogenosome-bearing ciliates do not form a monophyletic group, but instead have originated independently several times in ciliate evolution. This is further evidence favouring the hypothesis that ciliate hydrogenosomes evolved from mitochondria, as several convergent endosymbioses are conceivably unparsimonious (Embley *et al*., 1995).

More recently, definitive evidence for the mitochondrial origin of a ciliate hydrogenosome has been produced, as a genome has been discovered in the hydrogenosome of *Nyctotherus ovalis*. This was demonstrated by showing co-localisation of the staining pattern of an anti-hydrogenase antibody with an anti-DNA antibody within the hydrogenosome (Akhmanova *et al*., 1998). This evidence was corroborated by the amplification of a eubacterial-like SSU rRNA gene from several strains of *Nytotherus ovalis*, which branched with mitochondrial SSU genes from other ciliates in phylogenetic analyses (Hackstein *et al*., 1999). One would expect that this SSU rRNA would also localise to the hydrogenosome of *Nyctotherus* and that it would be encoded on the hydrogenosomal genome. However, the location of the SSU transcript is still unknown and it is not known which genes are encoded in the *Nyctotherus* hydrogenosomal genome. Characterisation of this genome promises to be fascinating, as other hydrogenosomes apparently do not possess genomes (Clemens and Johnson, 2000). It will be interesting to see what genes were retained and why the genome was maintained as the *Nyctotherus* mitochondrion was metabolically transformed (Palmer, 1997).

4.1.2. Chytrid hydrogenosomes

As with ciliates, most chytrid fungi are aerobic and contain mitochondria, but a small group of anaerobic chytrids is found in the alimentary tract of large herbivorous mammals, the best-studied being *Neocallimastix*. In the case of the chytrids, the debate has largely focused on whether

the hydrogenosome evolved from a mitochondrion or a peroxisome. The chytrid hydrogenosome was initially thought to be bound by a single membrane until it was shown to be double membrane-bounded using freeze fracture and standard electron microscopy using membrane preserving methods (Benchimol *et al*., 1997; van der Giezen *et al*., 1997). Thus, in this respect, it is morphologically similar to the mitochondrion and not the peroxisome. Peroxisomal targeting signals were thought to be present in *Neocallimastix* hydrogenase proteins based on cross-reactivity between this hydrogenosomal protein and an antibody raised to the peroxisomal targeting signal 1 (PTS1), a motif consisting of a C-terminal SKL motif (Marvin-Sikkema *et al*., 1993). This was interpreted as indicating an evolutionary relationship between the fungal hydrogenosome and peroxisome. However, sequencing of the *Neocallimastix* hydrogenase gene revealed that it did not encode an SKL motif, but rather an N-terminal extension resembling a targeting peptide (Davidson *et al*., 2002). Furthermore, *Neocallimastix* hydrogenosomal proteins have been shown to contain targeting peptides recognised by the yeast mitochondrial import system. When the yeast *Hansenula polymorpha* was transfected with the *Neocallimastix* hydrogenosomal malic enzyme, it was imported into the mitochondrion rather than the peroxisome (van der Giezen *et al*., 1998). Now, phylogenetic analyses have also shown that the *Neocallimastix* AAC transporter, a hydrogenosomal membrane protein responsible for the import of ADP and export of ATP, is closely related to the mitochondrial AAC carrier (van der Giezen *et al*., 2002). Taken together, the evidence strongly favours an hypothesis where the fungal hydrogenosome has in fact evolved from a mitochondrion.

4.1.3. Parabasalian hydrogenosomes

Parabasalia are a very large and extremely diverse group of protists, virtually all of which are found in some sort of association with animals. Most Parabasalia are innocuous or even essential to their hosts' survival (e.g., in the termite hindgut environment: Yamin, 1979), but others are important pathogens. *Trichomonas vaginalis* is a sexually transmitted human parasite infecting 170 million people each year and, as such, is the most common non-viral sexually transmitted disease. It causes genitourinary tract infections and increases susceptibility to HIV infection and cervical cancer, as well as increasing the risk of perinatal complications (Petrin *et al*., 1998). The current drug of choice for treatment is metronidazole. This drug's specificity relies on activation by the hydrogenosomal enzyme pyruvate: ferredoxin oxidoreductase (PFOR) (Müller and Lindmark, 1976).

The parabasalian hydrogenosome was the first to be characterised, and is currently the best studied in terms of biochemistry and cell biology. Initial reports suggested that parabasalid hydrogenosomes were bound by a single membrane (Lindmark and Müller, 1973), but it is now known that they are bound by a double membrane, as is the case with mitochondria (Benchimol and DeSouza, 1983). Furthermore, although one uncorroborated study did report circular DNA in trichomonad hydrogenosomes (Cerkasovova *et al*., 1976), it is now clear that those parabasalian hydrogenosomes that have been most closely examined lack DNA (Clemens and Johnson, 2000). The metabolism of parabasalian hydrogenosomes has been worked out in detail, and all the key metabolic enzymes from *T. vaginalis* have been characterised and their genes sequenced. These genes possess amino-terminal extensions that look something like mitochondrial targeting transit peptides, although they are by no means canonical (Bradley *et al*., 1997). Even with these data, however, the origin of the organelle was not entirely clear. Unlike the chytrids and ciliates, there was no certain indication that Parabasalia evolved from mitochondrial ancestors, so our understanding of the origin of the parabasalian hydrogenosome has been strongly influenced by our understanding of the evolutionary history of the host cell.

In contrast to ciliates and chytrids, parabasalids were once widely considered to be primitively amitochondriate organisms, members of the Archezoa, supposedly diverging from other eukaryotes prior to the endosymbiotic origin of mitochondria (Cavalier-Smith, 1983). Based on this perceived phylogenetic position, it was thought that the parabasalian hydrogenosome must have originated independently of the mitochondrion, either by an endogenous or alternative endosymbiotic origin. Indeed, even before the Archezoa hypothesis was formulated, it was suggested that hydrogenosomes might have originated from an endosymbiotic event with a *Clostridium*-like bacterium, as the enzymes PFOR and hydrogenase were not thought to occur in any other eukaryotes (Whatley *et al*., 1979).

In order to challenge the hypothesis that parabasalids originated before the mitochondrial endosymbiosis, and to see if an evolutionary link existed between parabasalian hydrogenosomes and typical mitochondria, molecular evidence for a cryptic mitochondrion was sought in the *Trichomonas* nuclear genome. Three chaperone genes were the targets of this search: *cpn*60, *hsp*70, and *hsp*10 (Bui *et al*., 1996; Germot *et al*., 1996; Horner *et al*., 1996; Roger *et al*., 1998). Recall that, in most eukaryotes, these genes are encoded in the nucleus, but their protein products are targeted to the mitochondrion. Phylogenetic analyses of these genes also show that they are closely related to alpha-proteobacterial homologues, as expected of mitochondrial genes. Accordingly, although none of these genes has been characterised in any mitochondrial genome, these genes are reasonably inferred to be derived

from the mitochondrial endosymbiont. All three genes were characterised in *Trichomonas*, and in phylogenetic analyses all three were found to cluster with their mitochondrial counterparts (e.g., Figure 4). This provided strong evidence that *Trichomonas* evolved from a lineage bearing a mitochondrion

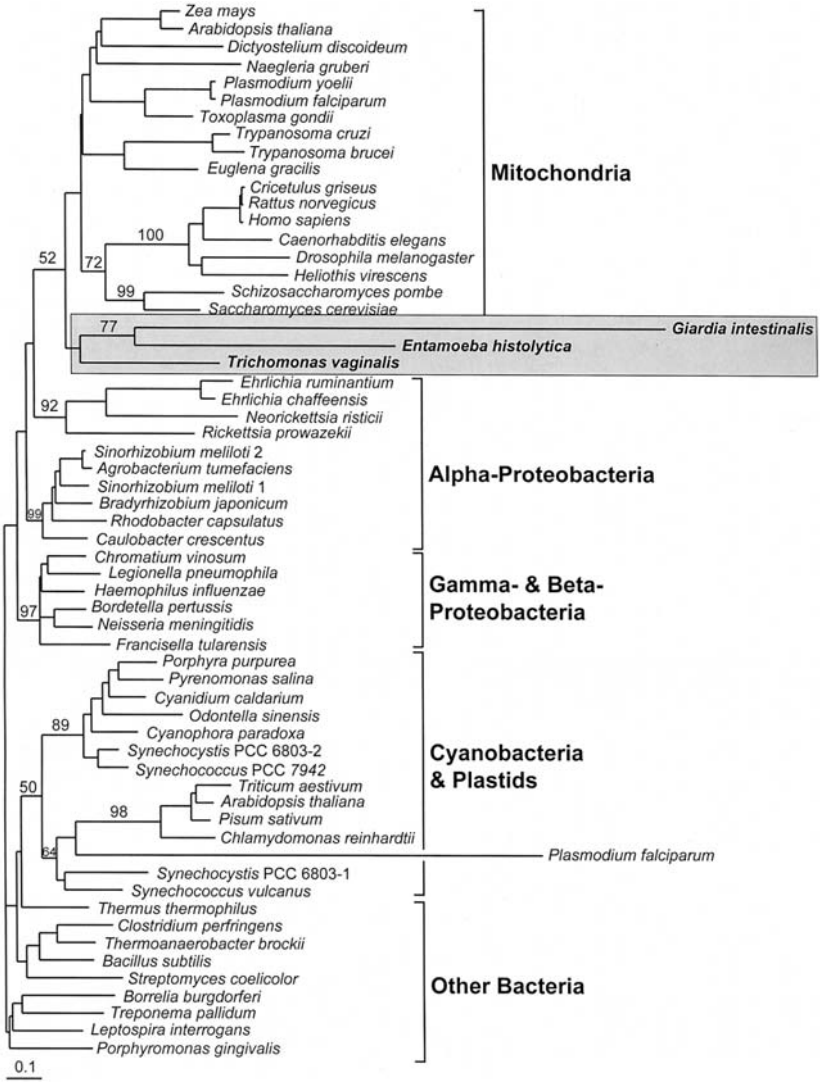

Figure 4 Phylogenetic tree of eubacterial, mitochondrial, and plastid chaperonin 60 (Cpn60). The tree is a weighted neighbour-joining tree (Bruno *et al.*, 2000) based

or mitochondrial ancestor, as these genes were interpreted as having been inherited from the mitochondrial endosymbiont and subsequently transferred to the nuclear genome, as in other eukaryotes. Ensuing research reinforced this conclusion by the characterisation of a mitochondrial-like pyridoxal-5′-phosphate-dependent cysteine desulfurase (IscS) from Parabasalia (Tachezy *et al*., 2001).

While the phylogenetic data argue very strongly for a mitochondriate ancestry of Parabasalia, they do not strictly address the origin of hydrogenosomes. This link was first established by demonstrating that the mitochondrial-like Cpn60 protein was co-extracted with proteins from hydrogenosomal fractions of *Trichomonas* cells, showing that the product of a gene of mitochondrial endosymbiont origin was localised to the hydrogenosome (Bui *et al*., 1996). Hydrogenosomal homologues of ADP-ATP carrier proteins were subsequently discovered and shown to be related to mitochondrial homologues. In addition, these proteins were shown to target yeast mitochondria in a manner similar in nature to the targeting of yeast homologues (Dyall *et al*., 2000). However, the most decisive evidence for a mitochondrial origin for the parabasalian hydrogenosome promises to come from the characterisation and analysis of certain elements of the protein-targeting machinery. Most proteins that have been analysed to date are derived from the genome of the alpha-proteobacterial endosymbiont, but some of the mitochondrial protein translocation factors were established only after the endosymbiosis. These proteins are not found in the bacterial antecedent of the organelle and are of host origin. The translocation system of *Trichomonas* is now being analysed biochemically, and distinctive features have been found in common with mitochondrial protein translocation (Bradley *et al*., 1997), suggesting that mitochondrion-specific proteins, such as the processing peptidase, may be present in *Trichomonas*. Characterising these proteins, and showing they are phylogenetically related to mitochondrial homologues, will provide even stronger evidence that the parabasalian hydrogenosome evolved from a fully integrated mitochondrion.

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on gamma-corrected distances (Strimmer and von Haeseler, 1996). Numbers at nodes refer to bootstrap support for that grouping, only high or important bootstrap values are given. Major lineages are bracketed and named to the right. The tree shows the mitochondrial homologues related to alpha-proteobacteria and plastid homologues related to cyanobacteria, as expected. The relatively divergent *cpn*60 genes from the erstwhile "amitochondriates" *Trichomonas*, *Entamoeba*, and *Giardia* (boxed and shaded) all branch with the mitochondrial clade, providing evidence for a mitochondrial ancestry of each of these protist parasites. Note also the plastid Cpn60 from the apicomplexan *Plasmodium*.

4.2. Entamoebid Mitosomes

The Archamoebae is a second group of purportedly amitochondriate protists proposed to belong to the Archezoa. Archamoebae encompasses the amitochondriate amoebae *Entamoeba*, *Pelomyxa* and mastigamoebids, of which the human parasite *Entamoeba histolytica* is the best studied. These groups were assumed to represent another ancient eukaryotic lineage on the basis of their "primitive" features: the absence of an identifiable mitochondrion, peroxisomes or Golgi dictyosomes (Cavalier-Smith, 1991a), and their "bacterial-like" fermentative metabolism using the enzymes PFOR, ferredoxin, and alcohol dehydrogenase (Reeves, 1984; Müller, 1992). This primitive cell structure led to their inclusion within the Archezoa, but they were soon removed from this group as SSU rRNA phylogenies showed that *Entamoeba* diverged from other eukaryotes after several mitochondrial groups. This position was debated extensively by comparing phylogenies based on several different molecular markers (Hasegawa *et al*., 1993; Leipe *et al*., 1993), and there was even considerable evidence that the various groups of Archamoebae were not related to one another (for review see Keeling, 1998). Now there is a growing consensus that Archamoebae branch with certain other amoebae and slime moulds at some position near the divergence of animals and fungi (Bapteste *et al*., 2002). Interestingly, recent data on the root of the eukaryotic tree have brought this debate full circle in one sense, by leading to the revival of the notion that Archamoebae are near the base of eukaryotes (Stechmann and Cavalier-Smith, 2002). Even while the position of *Entamoeba* was under scrutiny, it was assumed to lack mitochondria, and that a mitochondriate origin for the Archamoebae only meant that the organelle had been lost secondarily. However, suggestions of the presence of a mitochondrion can be found in early *Entamoeba* literature: mitochondria-like organelles were found through the use of vital stains, and the activity of the mitochondrial enzyme pyridine nucleotide transhydrogenase (PNT) had been demonstrated (Causey, 1925; Harlow *et al*., 1976). Subsequently, mitochondrial gene sequences were sought and found in the nuclear genome of *Entamoeba*, proving its mitochondrial ancestry decisively. In this case, gene sequence from PNT and mitochondrial *cpn*60 were initially characterised (Clark and Roger, 1995), and both were shown to be closely related to mitochondrial homologues in phylogenetic analysis (e.g., Figure 4). This was recently supported by the characterisation of a mitochondrial HSP70 in *Entameoba* (Bakatselou *et al*., 2000). Interestingly, all three genes encode N-terminal extensions with some similarity to transit peptides (Clark and Roger, 1995; Bakatselou and Clark, 2000), indicating the possible

presence of mitochondrial-targeting peptides, fueling the idea that a remnant mitochondrion may still be present in *Entamoeba*.

This possibility was tested by localisation studies using the Cpn60 protein – the protein with the most compelling evidence for mitochondrial ancestry from phylogenetics. This protein was independently shown by two groups to localise to a relatively nondescript, double membrane-bounded structure (Mai *et al*., 1999; Tovar *et al*., 1999). In one study, cells were transfected with a mtCpn60-c-*myc* fusion protein, and the protein was shown to be localised to a single 1 μm diameter structure using anti-c-myc antibodies (Tovar *et al*., 1999). When the first 15 amino acids of the sequence were removed, it was shown that localisation was disrupted, but then restored by the addition of a trypanosome mitochondrial transit peptide. A second study showed that antibodies raised directly against the *Entamoeba* Cpn60 localised to a small, low copy number organelle in a similar pattern (Mai *et al*., 1999). This cryptic mitochondrion was dubbed either the mitosome or crypton (Mai *et al*., 1999; Tovar *et al*., 1999). Interestingly, the mitosome has also been shown to stain with the DNA-binding fluorochromes, propidium iodide, sytox green, and acridine orange, which has been taken to mean that it has retained a genome (Ghosh *et al*., 2000). Whether this does represent the presence of DNA and, if so, exactly what this genome would encode, are both intriguing questions that await molecular characterisation. It is also difficult to predict the possible function of the organelle; presently Cpn60 is the only protein that has been localised to the structure, although it seems very likely that PNT and HSP70 are also mitosomal based on their phylogenetic positions. In addition, PFOR has been reported to be associated with discrete cytoplasmic structures, possibly mitosomes (Rodriguez *et al*., 1998). In contrast, two other key enzymes in the metabolism of *Entamoeba*, ADH and ferredoxin, have been demonstrated to reside in the cytosol (Mai *et al*., 1999). This story is obviously very complex, and only a small part of the picture is available at present. Nevertheless, it seems that the core carbon metabolism of *Entamoeba* is not compartmentalised to the extent that is seen in parabasalian hydrogenosomes, and that the mitosome is probably retained for a limited number of functions, whatever they may be.

4.3. Microsporidian Mitosomes

Microsporidia are intracellular parasites that infect a broad range of animals, including immunocompromised humans, as well as a few species of ciliate and gregarine Apicomplexa. In humans, they cause a variety of ailments, most commonly diarrhoea, but also keratitis, sinusitis,

encephalitis, and myositis (Weber *et al*., 1994). The extracellular spore stage of microsporidia is dominated by a highly specialised and unique infection apparatus (Vávra and Larsson, 1999). Most obvious is the polar tube, which is used to pierce host cells and transmit the parasitic sporoplasm to the cytosol of the host. In contrast to the highly ordered and easily recognisable spore, electron microscopy of meront stages of microsporidia, an intracellular stage representing most of the actively dividing microsporidia, shows these cells to be amorphous with few obvious ultrastructural features, lacking mitochondria, centrioles, peroxisomes, and microtubules (Vávra and Larsson, 1999).

The apparent simplicity of microsporidia was interpreted as primitive, and thus microsporidia were also included in the Archezoa and considered to be primitively amitochondriate (Cavalier-Smith, 1983). The first molecular phylogenies based on SSU rRNA, together with a prokaryotic-like fused 5.8S-LSU rRNA structure, supported the hypothesis that microsporidia were one of the first branches of the eukaryotic tree (Vossbrinck and Woese, 1986; Vossbrinck *et al*., 1987). Ensuing phylogenies of microsporidian EF-1α and EF-2 genes further substantiated this position (Kamaishi *et al*., 1996a, b), and established microsporidia as a ''textbook'' example of early branching eukaryotic cells (Tudge, 2000; Madigan *et al*., 2002). However, rigorous phylogenetic analyses based on several protein-coding genes have now shown microsporidia to be related to fungi, and not at all ancient (Edlind *et al*., 1996; Keeling and Doolittle, 1996; Fast *et al*., 1999; Hirt *et al*., 1999; Keeling *et al*., 2000; Van de Peer *et al*., 2000; Keeling, 2003). As fungi are clearly mitochondriate, microsporidia must have evolved from a mitochondriate ancestor, and either lost the organelle or retained a cryptic mitochondrion. The first direct evidence for a mitochondriate ancestry came, once again, from genes encoding mitochondrial HSP70 (e.g., Figure 5). This mitochondrion-derived gene was characterised from the nuclear genomes of several species of microsporidia (Germot *et al*., 1997; Hirt *et al*., 1997; Peyretaillade *et al*., 1998), and was soon supported by the characterisation of α and β subunits of mitochondrial pyruvate dehydrogenase (PDH) (Fast and Keeling, 2001), putting to rest the idea that microsporidia were primitively amitochondriate (Figure 6). The complete sequence of the *E. cuniculi* genome added several more putative mitochondrial genes to this list, some of which have interesting implications. In addition to HSP70 and the two subunits of PDH, the *Encephalitozoon* genome was found to encode homologues of TIM22, TOM70, Erv1p, Atm1p (a homologue of the ABC transporter), ferredoxin (Yah1p), manganese superoxide dismutase (MnSOD), Nifu1p-like protein, frataxin (Yfh1p), and mitochondrial glycerol-3-phosphate dehydrogenase (Katinka *et al*., 2001). This extensive list allowed Katinka *et al*. (2001) to hypothesise

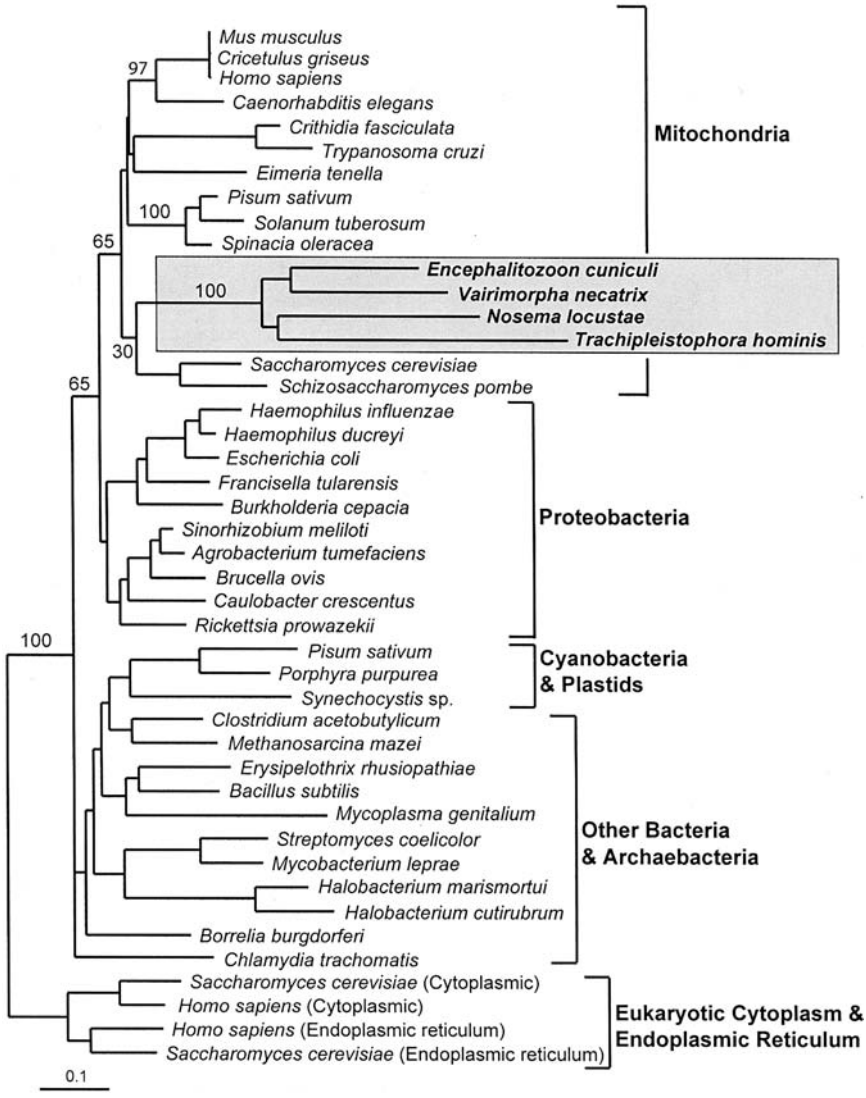

Figure 5 Phylogenetic tree of eubacterial, mitochondrial, and plastid HSP70 proteins. The tree is constructed in the same way as that shown in Figure 4. Note that the sequences from four microsporidian sequences (boxed and shaded) all branch within the mitochondrial clade, and branch weakly with fungi.

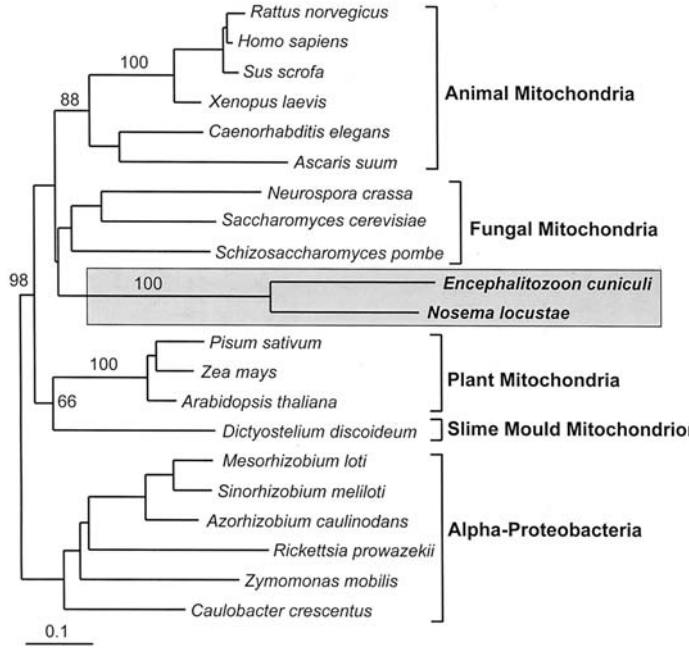

Figure 6 Phylogenetic tree of the beta subunit of pyruvate dehydrogenase (PDH) E1 from alpha-proteobacteria and mitochondria. The tree is constructed in the same way as that shown in Figure 4. Note that the microsporidian sequences (boxed and shaded) are related to mitochondrial homologues, and branch weakly with the fungi. This tree is an example of how phylogeny can be used to show the mitochondrial origin of a protein in microsporidia.

the existence of a remnant mitochondrion-derived organelle in microsporidia, which they called a mitosome. However, there was still no physical evidence for the presence of an organelle, and analysis of the N-termini of putatively mitochondrial proteins from microsporidia led to contradictory conclusions (Fast and Keeling, 2001; Katinka *et al.*, 2001), so it remained uncertain whether microsporidia had actually retained a mitochondrial organelle. The production of a specific antibody to the mitochondrial HSP70 from the microsporidian *Trachipleistophora hominis* allowed the localisation of the first mitochondrion-derived protein in microsporidia (Williams *et al.*, 2002). The HSP70 was found to localise to double membrane-bounded structures, which are abundant but extremely small, at just 90 nm at their broadest. These structures are relict mitochondria-derived organelles, and probably represent the

smallest mitochondria presently known (Williams *et al*., 2002). Even in the smallest eukaryote, *Ostreococcus tauri*, a picoplanktonic alga, which measures a mere 1.5 μm in diameter, mitochondria are twice the size of the structures seen in the microsporidian cells (Chretiennot-Dinet *et al*., 1995).

Like other putatively mitochondrial proteins in microsporidia, mitochondrial-targeting signals could not be detected in the *T. hominis* HSP70 sequence. This is unlike the situation in trichomonad hydrogenosomes or *Entamoeba* mitosomes, where recognisable mitochondrial-targeting signals have been retained. Despite this, the *T. hominis* HSP70 protein is apparently successfully translocated across the organelle's double membrane into the matrix. This raises interesting questions regarding the nature of the import system present in the microsporidian mitochondrial-derived organelle. It is clearly homologous to other mitochondrial-targeting systems, since the *Encephalitozoon* genome encodes homologues of mitochondrial translocation complex proteins (e.g., TIM22, TOM70), but the system is also clearly different. Most of the translocation complex homologues have not been detected in the *Encephalitozoon* genome (including a stromal peptidase), and the transit peptides are typically very reduced or even absent, as discussed above (although some genes have very long leaders that do resemble transit peptides, e.g., glycerol-3-phosphate dehydrogenase). Microsporidia are well known to have streamlined much of their biology in adapting to their parasitic lifestyle (Keeling, 2001; Keeling and Fast, 2002) and it is possible that this process has extended to the mitochondrial protein import machinery, resulting in a unique system that could potentially no longer rely on cleaved N-terminal transit peptides.

The possible functions of this organelle also remain somewhat unclear, although the complete genome of *Encephalitozoon* certainly allows for informed speculation. As microsporidia are anaerobes and have previously been shown to lack oxidative phosphorylation and TCA cycle (Weidner *et al*., 1999), none of the proteins related to these pathways are found in the genome. A high proportion (five) of the identified mitochondrial proteins retained by *E. cuniculi* is involved in iron–suphur cluster assembly, making this a leading candidate function for the highly reduced organelle (Katinka *et al*., 2001). In addition, the presence of PDH subunits is interesting. In typical mitochondria, PDH complex (composed of PDH E1, E2, and E3) catalyses the first step in the breakdown of pyruvate, resulting in acetyl-CoA which is passed into the TCA cycle (Pronk *et al*., 1996). In the ''amitochondriates'' like *Trichomonas*, *Entamoeba*, and *Giardia*, PDH is absent and pyruvate is activated by PFOR (Müller, 1992), the target for the most widely used drugs against these parasites. Microsporidia appear to lack PFOR, contain PDH E1, but lack E2 and E3 (Fast and Keeling, 2001;

Katinka *et al*., 2001). They also contain ferredoxin, and ferredoxin:NADH oxidoreductase (Katinka *et al*., 2001). Taken together, this suggests that there is some energy-generating metabolism of pyruvate taking place in the mitosome, but of a unique nature; resembling neither typical mitochondrial metabolism or the metabolism that has been characterised in other ''amitochondriates''.

4.4. Mitochondria in Diplomonads?

The diplomonads, of which *Giardia intestinalis* (syn. *lamblia*) is the best studied member, are a fairly small group of anaerobes and microaerophiles that are predominantly parasites of a variety of animals, but also include some free-living genera (Brugerolle and Lee, 2002). Diplomonads are another textbook example of ''primitive'' amitochondriate eukaryotes, often lauded as the first lineage of eukaryotes (Sogin *et al*., 1989; Sogin and Silberman, 1998). This evolutionary position has been substantiated by many molecular phylogenies (Sogin *et al*., 1989; Hashimoto *et al*., 1994, 1995), but has also come under scrutiny in recent years because of evidence that other purportedly ''deep'' lineages are really misplaced long-branches in the eukaryotic tree, and the observation that diplomonad sequences are also generally very divergent (Embley and Hirt, 1998). Like *Trichomonas* and *Entamoeba*, *Giardia* relies on anaerobic rather than aerobic energy metabolism and uses the key enzyme PFOR. Indeed, the metabolism characterised in *Giardia* is very similar to that of *Entamoeba* (Müller, 1992), despite clear evidence that the two are very distantly related. The important metabolic enzymes that have been studied in *Giardia* have not been shown to act within a specialised compartment. Diplomonads were also considered to be Archezoa, and accordingly thought to have never had a mitochondrion (Cavalier-Smith, 1983). However, as for *Trichomonas*, microsporidia, and *Entamoeba*, molecular phylogenetic evidence for a mitochondrial ancestry has been found in diplomonads.

The evidence for a mitochondriate origin of diplomonads actually goes back some time, but the early evidence was weak and, in retrospect, probably not accurately interpreted. The first such evidence came from phylogenies of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Although GAPDH does not act in the mitochondrion of modern eukaryotes (with one very odd exception: the diatom *Phaeodactylum tricornutum*: Liaud *et al*., 2000), phylogenies were interpreted as showing that the eukaryotic enzyme was derived from the mitochondrial endosymbiont (Martin *et al*., 1993). The *Giardia* GAPDH was like other eukaryotic homologues, and it was therefore interpreted as

evidence for a mitochondrial ancestry of diplomonads. However, the mitochondrial origin of eukaryotic GAPDH genes has not withstood further phylogenetic analysis (Kurland and Andersson, 2000), so the *Giardia* GAPDH does not likely reflect a mitochondriate origin of diplomonads after all. Similar arguments have been made for another glycolytic enzyme, triosephosphate isomerase (TPI) (Keeling and Doolittle, 1997). Once again, however, the mitochondrial origin of TPI has not been substantiated by further analysis (Kurland and Andersson, 2000).

Much stronger evidence for a mitochondriate ancestry of diplomonads came from the dual-function valyl-tRNA synthetase of eukaryotes (which functions in both the cytosol and mitochondrion). Phylogenetic analysis suggested that this enzyme was also derived from the mitochondrial endosymbiont (Brown and Doolittle, 1995), including the *Giardia* homologue (Hashimoto *et al*., 1998). This case is stronger than either TPI or GAPDH, but still suffers from a somewhat indirect connection to the mitochondrion. More direct evidence for mitochondria was sought, as in other Archezoa, using the mitochondrial chaperonin, Cpn60. In contrast to the chain of discoveries in other Archezoa, however, the first data for putative mitochondrial chaperonins in *Giardia* came from localisation experiments using heterologous antibodies to Cpn60 raised against human or rodent mitochondrial Cpn60, or prokaryotic Cpn60. Labelling of *Giardia* cells with such heterologous antibodies suggested that a prokaryotic Cpn60 was present in the cell and was localised in a punctate fashion, but not to discrete compartments (Soltys and Gupta, 1994). Subsequently, genes for *cpn*60, *hsp*70, and IscS were characterised in *Giardia*, and were shown to be phylogenetically related to mitochondrial homologues (Roger *et al*., 1998; Morrison *et al*., 2001; Tachezy *et al*., 2001), confirming that the diplomonads did have a mitochondriate ancestry (e.g., Figure 4).

However, the presence or absence of a mitochondrion has not yet been rigorously tested in *Giardia*, and at present available evidence is conflicting. First, none of the proteins shown to be phylogenetically derived from the mitochondrial endosymbiont encodes leaders that could unambiguously be interpreted as transit peptides (Roger *et al*., 1998; Horner and Embley, 2001; Morrison *et al*., 2001; Tachezy *et al*., 2001). Moreover, localisation data from heterologous antibodies to Cpn60 give slightly different results depending on the antibody used, although none have produced convincing evidence for an organelle (Soltys and Gupta, 1994; Roger *et al*., 1998). Similarly, thorough inspection of electron micrographs of *Giardia* has not shown the presence of a typical mitochondrion, although early literature on *Giardia* did document sightings of structures which may resemble reduced mitochondria, and hypothesised that their unusual structure may be due to the low oxygen environment to which *Giardia* is adapted (Cheissin, 1965).

Nevertheless, areas of the *Giardia* cell have also been shown to react with CTC, a marker of respiratory chain activity. Similarly, the membrane potential detecting dye, Rhodamine 123, also labelled isolated zones, indicating discrete, membrane-bounded areas within the cell with an electron potential (Lloyd *et al*., 2002).

Interestingly, a little-studied flagellate, *Carpediemonas membranifera*, has recently been described and shown to have a membrane-bounded organelle reminiscent of a hydrogenosome, although there are no biochemical data on the function of this organelle (Simpson and Patterson, 1999). The phylogenetic position of *Carpediemonas* has now been analysed using alpha-tubulin, beta-tubulin, and SSU rRNA, and intriguingly it was found to be strongly supported as the sister group to retortamonads and diplomonads (Simpson *et al*., 2002). This free-living flagellate has the potential to hold many of the answers to the evolutionary fate of the mitochondrion in diplomonads like *Giardia*, but it will be difficult to draw any conclusions until the *Carpediemonas* organelle is better characterised. Until then, the data for or against an organelle in *Giardia* remain inconclusive, and if such an organelle does exist, its function is entirely unclear. The characterisation of iron–sulphur cluster assembly proteins (Tachezy *et al*., 2001) may indicate that, as in other eukaryotes, iron–sulphur centres are created within the mitochondrion and exported for use in cytosolic iron–sulphur proteins. However, this is very speculative as definitive evidence for an organelle is still lacking and, by extension, no protein has been localised to an organelle in *Giardia*, both of which are necessary to understand the function of a cryptic organelle.

5. CASE HISTORIES – PLASTIDS

5.1. Apicomplexa

The Apicomplexa is a phylum entirely composed of obligate intracellular parasites, some of which cause extremely important diseases of both medical and commercial concern. The most serious of these is malaria, caused by members of the genus *Plasmodium*, which remains one of the most deadly infectious diseases throughout much of the world today.

In the mid-1970s, electron microscopic observations of the extrachromosomal DNA of an avian malaria parasite revealed the presence of a circular element that contained a cruciform structure indicative of an inverted repeat (Kilejian, 1975). This genome was reasonably interpreted as the mitochondrial genome (Kilejian, 1975; Gardner *et al*., 1988), but subsequent

sequencing of genes from this element revealed that, although they were eubacterial in nature, they did not bear any particular resemblance to homologues from other mitochondrial genomes. Instead, and surprisingly, these genes were found to be most similar to homologues from plastid genomes (Gardner *et al*., 1991, 1993), a finding corroborated by the discovery of certain genes that are never found in other mitochondrial genomes, but are common to plastid genomes (Gardner *et al*., 1994; Williamson *et al*., 1994).

Overall, the characteristics of this genome led Iain Wilson and colleagues to propose that the 35 kb circle, as it was known, was a plastid genome (Gardner *et al*., 1991). This rather unusual idea was significantly supported by the discovery and characterisation of a second extrachromosomal element in Apicomplexa. This was a 6 kb linear element that encoded genes for *cox*I, *cox*III, and *cob*, all mitochondrial proteins, as well as fragments of mitochondrial rRNA genes (Vaidya *et al*., 1989; Feagin *et al*., 1991). This small and unusual element was shown to co-fractionate with the mitochondrion (Wilson *et al*., 1992), revealing it to be the most reduced and odd of all mitochondrial genomes known (Gray *et al*., 1999).

Ultimately, the plastid nature of the 35 kb circle was made crystal clear by the complete sequencing of the *Plasmodium falciparum* element (Wilson *et al*., 1996), and later that of *Toxoplasma gondii* (Köhler *et al*., 1997). Although the genome was reduced and obviously lacked any genes directly related to photosynthesis, the complete sequence revealed a number of characteristics known to be unique to plastid genomes. These include the rRNA operon inverted repeat (which was originally observed by electron microscopy), and the plastid "superoperon (Wilson *et al*., 1996). At the same time, plastid genomes were also shown to be present in a diversity of Apicomplexa (Wilson *et al*., 1996; Lang-Unnasch *et al*., 1998), showing that the genome is a widespread characteristic of the group.

The actual organelle was identified by localising transcripts from plastid-encoded SSU rRNA (McFadden *et al*., 1996), and by localising the genome itself (Köhler *et al*., 1997). Not surprisingly, the plastid bears little resemblance to the canonical chloroplast; instead, it is a relatively non-descript multimembrane structure typically found once per cell. The first description of the *Toxoplasma* plastid suggested it was surrounded by two or perhaps three membranes (McFadden *et al*., 1996), however it was later shown to be a four-membrane structure (Köhler *et al*., 1997), suggesting a secondary origin (see Figure 2). This has subsequently come under dispute once again with the suggestion that there are actually three plastid membranes (Hopkins *et al*., 1999), but this has not been corroborated. Interestingly, the structure finally identified as the plastid had been documented on several occasions and given several different names by

earlier electron microscopists (McFadden and Waller, 1997). Understandably, none had guessed its origin or had any idea of its function.

Indeed, the function of the organelle was still unclear even after both the *Plasmodium* and *Toxoplasma* plastid genomes had been completely sequenced: both genomes contained a large number of genes encoding proteins involved in gene expression, but none that stood out as obviously functionally critical genes that could explain the retention of the organelle (Wilson *et al*., 1996; Köhler *et al*., 1997). These obligate intracellular parasites are obviously not photosynthetic, so what does a plastid do in Apicomplexa? It was shown very early on that the plastid was essential to the parasites, although it is interesting to note that knocking out plastid translation did not kill the parasites immediately, but disrupted subsequent infection cycles (Fichera and Roos, 1997; He *et al*., 2001). The answer to this puzzle lay in the biochemical flexibility of plastids, and the nuclear genome of the parasites. In plants and algae, plastids are nearly always thought of in association with photosynthesis, as this is the major biochemical activity of the organelle and the characteristic that most obviously sets these organisms apart from other eukaryotes. However, plastids are more than simply photosynthetic sugar factories. In addition to photosynthesis, and the biochemical pathways responsible for synthesising the chemical components necessary for photosynthesis, plant and algal plastids have a number of other important jobs. The best understood are the synthesis of fatty acids, isopentenyl diphosphate (the core unit of isoprenoid compounds), heme, and some amino acids, although a number of other important roles are also known (Herrmann, 1995; Harwood, 1996; Rohdich *et al*., 2001). Recall that, during the early stages of endosymbiosis, most of the plastid genes were moved to the host nucleus, with their products being post-translationally targeted back to the organelle. Accordingly, the majority of the functionally critical proteins are encoded by plastid-derived genes in all plants and algae, but many of these genes are nucleus-encoded. Searching the nuclear genomes of *Plasmodium* and *Toxoplasma* soon revealed the presence of a number of genes for plastid-targeted proteins, which were identified based on their phylogenetic relationship with plastid and cyanobacterial homologues (e.g., Figure 7). The first such proteins to be identified were involved in fatty acid biosynthesis (Waller *et al*., 1998). These proteins were shown to be localised to the plastid by immunocytochemistry, and to encode N-terminal leader peptides with signal and transit-peptide domains (as expected for a secondary plastid) that were sufficient to target Green Fluorescent Protein (GFP) to the plastid in transfected cells (Waller *et al*., 1998). Subsequently, the nuclear genome has also been found to harbour genes for plastid-targeted proteins making up complete pathways for fatty acid, isopentenyl diphosphate, and heme biosynthesis (Waller *et al*., 1998;

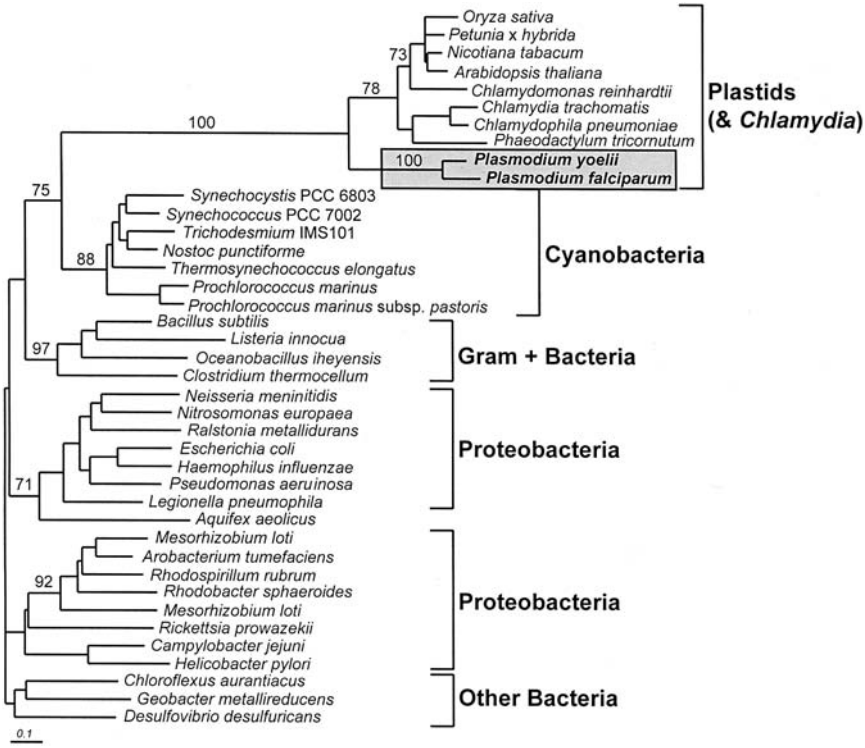

Figure 7 Phylogenetic tree of eubacterial and plastid FabI (enoyl-ACP reductase). The tree is constructed in the same way as that shown in Figure 4. The (divergent) plastid homologues branch with the cyanobacteria, as expected, and the *Plasmodium* genes (boxed and shaded) branches within the plastid with very high statistical support. Curiously, the chlamydial FabI appears to be derived from a plastid gene, which has been noted previously. This tree is an example of how phylogeny can be used to show the plastid origin of a protein in *Plasmodium*, and shows that Type I fatty acid biosynthetic enzymes are plastid-derived in Apicomplexa.

Jomaa *et al.*, 1999; Gardner *et al.*, 2002; Sato and Wilson, 2002). The Apicomplexa also have genes for aromatic amino acid biosynthesis (Roberts *et al.*, 1998), but these are not plastid derived (Keeling *et al.*, 1999) and are not localised to the plastid (Fitzpatrick *et al.*, 2001). The biosynthesis of fatty acids, isopentenyl diphosphate, and heme can explain why the Apicomplexa retains a plastid. When the plastid originated in this lineage, it assumed essential functions other than photosynthesis (either these

pathways did not exist in the pre-plastid host, or they were made redundant by the plastid pathway and were lost in the host). As the ancestors of Apicomplexa progressively adapted to intracellular parasitism and the plastid lost its role in photosynthesis, it could not simply be lost unless another source of these essential compounds could be secured. As parasites, the Apicomplexa absorbs many nutrients from their hosts, but apparently the compounds synthesised by the plastid are either not acquired from the host at some stage of the life cycle, or some particular variants need to be synthesised for specialised purposes.

At about the time that the first sequences from the apicomplexan plastid genome became available, the evolutionary position of the Apicomplexa was also being examined using nuclear gene sequences. Apicomplexa had been classified for convenience (Kudo, 1947) with other "spore-forming parasites" such as microsporidia and actinosporidia (apicomplexans do not form spores). The phylogenies of nuclear SSU and 5S rRNA, however, showed a very strongly supported relationship between Apicomplexa, ciliates, and dinoflagellate algae (Gajadhar *et al*., 1991; Wolters, 1991). Subsequent phylogenies based on a variety of protein-coding genes have lent further strong support for this group (Fast *et al*., 2002; Saldarriaga *et al*., 2003), and ultrastructural studies have revealed that these three lineages share a number of structural features. This group is now known as Alveolata, and is one of the best-supported major protist groupings presently defined. Within the alveolates, the Apicomplexa appear to be more closely related to the dinoflagellates than either are to ciliates (Fast *et al*., 2002). Dinoflagellates are a very diverse group of predators, parasites, and most interestingly, phototrophs. About one half of the described dinoflagellates are photosynthetic (Taylor, 1987), and their plastids were acquired by secondary endosymbiosis with a red alga (Zhang *et al*., 2000).

The relationship between Apicomplexa and dinoflagellates led to obvious questions as to whether their plastids arose from a single common endosymbiosis in their ancestor, or if they arose independently by two secondary endosymbiotic events. The former alternative requires fewer independent plastid origins, and indeed, it has been suggested that the plastid in both groups arose much earlier in a common ancestor of all alveolates and a number of other groups with secondary plastids, namely heterokonts, haptophytes, and cryptomonads (Cavalier-Smith, 1999). On the other hand, no plastids have been found in the apparent deep diverging lineages of both dinoflagellate and apicomplexan lineages (Saldarriaga *et al*., 2001; Kuvardina *et al*., 2002), so it was also reasonable to assume that each group acquired its plastid individually and recently (Taylor, 1999). In addition, there has been some debate over whether the apicomplexan plastid was derived from a red or a green alga. In favour of a red algal origin, the

plastid genome bears some characters in common with red algal plastid genomes, most notably the structure of the plastid super-operon, and several genes have also shown weak support for a red algal affinity (Gardner *et al*., 1994; McFadden and Waller, 1997; Blanchard and Hicks, 1999). In contrast, the phylogeny based on the plastid *tuf*A gene weakly showed a green algal affinity (Köhler *et al*., 1997). A green algal origin of the apicomplexan plastid is inconsistent with a single early origin for apicomplexan and dinoflagellate plastids since the ancestral dinoflagellate plastid is clearly derived from a red alga by secondary endosymbiosis (Zhang *et al*., 2000). The obvious way to solve each of these problems is to infer phylogenetic trees using plastid gene sequences to see if the dinoflagellates and Apicomplexa form a unique clade nested within the red algae. Plastid gene sequences from dinoflagellates were only recently characterised, and were found to reside on single gene mini-circles rather than the expected single circular chromosome as found in other plastids (Zhang *et al*., 1999). Unfortunately, nearly all genes found in dinoflagellate plastids to date have encoded proteins specific for photosynthetic functions (Zhang *et al*., 1999; Barbrook and Howe, 2000; Barbrook *et al*., 2001; Hiller, 2001), so there is little plastid information to compare between Apicomplexa and dinoflagellates. To make matters worse, both apicomplexan and dinoflagellate plastid genes are very divergent, which can potentially lead to difficulties in phylogenetic inferences (Zhang *et al*., 2000). Nonetheless, in gene trees based on plastid SSU and LSU rRNA (the only comparable genes known today), the apicomplexan and dinoflagellates form a clade (Zhang *et al*., 2000). In contrast to the plastid-encoded genes, genes for plastid-targeted proteins in both groups appear to be relatively conserved, leading to a large source of potential genes for comparison. The first (and presently the only) plastid-targeted gene to be sampled from both groups is GAPDH. GAPDH is found in both the cytosol, where it is largely NADH-dependent and catabolic, and the plastid, where it is either NADH or NADPH-dependent, and anabolic. In plants, green algae, red algae, and *Euglena*, the cytosolic gene is related to other eukaryotic GAPDH genes and the plastid gene is related to cyanobacterial homologues, as expected. However, in Apicomplexa and dinoflagellates, both GAPDH homologues are related to eukaryotic cytosolic genes, and most importantly, both the cytosolic and plastid genes from both groups form clades and are each most closely related to one another than to any other GAPDH (Fast *et al*., 2001). The situation in dinoflagellates is perhaps more complex, since putative plastid-targeted genes resembling canonical plastid-targeted genes have also been described (Fagan and Hastings, 2002). Where these genes came from is not certain, but it appears most likely that the cytosolic GAPDH in some ancestor of Apicomplexa and dinoflagellates duplicated and the protein

product of one copy was targeted to the plastid where it took over the function of the existing cyanobacterium-derived plastid gene. This kind of event, called "endosymbiotic gene replacement" is known to happen (e.g., Brinkmann and Martin, 1996), but is very unusual and distinctive. Such a rare event, together with the high degree of similarity between the plastid homologues from both groups, provides very strong evidence that the plastids found in these two lineages originated by a single secondary endosymbiosis in a common ancestor of Apicomplexa and dinoflagellates. More interesting still, it has been shown that other photosynthetic groups with red algal secondary endosymbionts, namely cryptomonads, heterokonts (Fast *et al*., 2001) and haptophytes (J. T. Harper and P. J. Keeling, unpublished data), also acquired their plastid GAPDH from this gene duplication. Thus, this plastid was transferred in the common ancestor of all these groups, long before the origin of Apicomplexa (Figure 3). This finding has significant impact on our understanding of a number of other parasites. The human parasite *Blastocystis* and oomycete parasites such as *Pythium* and *Phytopthora*, belong to the heterokonts (Nakamura *et al*., 1996; Arisue *et al*., 2002). These have not traditionally been considered to possess a plastid, but the ancient origin of the plastid in their photosynthetic relatives suggested by the GAPDH data implies that these organisms might indeed possess cryptic plastids (see Andersson and Roger, 2002). To date no direct evidence for such an organelle exist, but this is not surprising since little molecular data are known for most of these organisms.

The single, early, red algal origin of the plastids in Apicomplexa and other chromalveolates has recently been challenged once again by the finding of a curious split CoxII in Apicomplexa. CoxII is a mitochondrial protein that is encoded in the mitochondrial genome of most eukaryotes, but in certain green algae the gene has been transferred to the nucleus and is split into two subunits, *cox*2a and *cox*2b (Perez-Martinez *et al*., 2001). Interestingly, the genomes of *Plasmodium* and *Toxoplasma* both encode a similarly split form of *cox*2, and it has been argued that this demonstrates that the apicomplexan plastid is derived from a green alga (Funes *et al*., 2002). While this certainly is an intriguing discovery, whether it relates to the origin of the plastid is far from obvious. For these two *cox*2 subunits to have originated from a green algal endosymbiont, they would have had to have been transferred from the nuclear genome of the endosymbiont to the nuclear gene of the secondary host, presumably replacing an ancestral gene encoded in the mitochondrial genome. This is not impossible to imagine, but the complete genome of *Plasmodium* has not been reported to encode any green algal genes other than the two *cox*2 subunits (Gardner *et al*., 2002), begging the important question, "why were both subunits of *cox*2 transferred, but no other green algal genes?" Moreover, the relationship

between Apicomplexa and other alveolates is now irrefutable. The evidence for the close relationship between heterokonts, haptophytes and cryptomonads (Yoon *et al*., 2002), and their relationship to alveolates is growing stronger as molecular data continue to accumulate (Baldauf *et al*., 2000; Fast *et al*., 2001). This nests the Apicomplexa phylogenetically within a group of protists with secondary endosymbionts of clear red algal ancestry (Figure 3). Accordingly, the conclusion that apicomplexan plastids came from the same endosymbiosis as other chromalveolate plastids, strongly supported by GAPDH (Fast *et al*., 2001), requires no special or spectacular events. In contrast, a green algal origin of the apicomplexan plastid requires more explanation. One suggestion is that an ancestral red algal plastid was replaced with a new green one (Palmer, 2003). This kind of event has happened several times in dinoflagellates (Tengs *et al*., 2000; Saldarriaga *et al*., 2001), and is therefore a plausible explanation where both gene trees are correct. However, given that the plastid genome itself retains a few characteristics suggesting a red algal origin, the possibility that the *cox*2 data are misleading should be considered seriously. Indeed, a recent reanalysis of *cox*2 including the phylogenetically critical genes from ciliates showed that the apicomplexan and ciliate *cox*2 genes were closely related, as expected if no lateral transfer took place (R. F. Waller, P. J. K., G. van Dooren, and G. I. McFadden, unpublished results). Moreover, the ciliate *cox*2 gene contains a 300 amino acid insertion and exactly the position where the split took place, and hydrophobicity analyses suggest that the splitting of the *cox*2 protein might have occurred twice independently to allow the protein to be translocated across the mitochondrial membrane. Altogether, we consider it unlikely that apicomplexan and green algal *cox*2 genes share a close common ancestor, despite their shared structural characteristics.

The discovery of a relict plastid in Apicomplexa is of tremendous interest for reasons relating to basic questions of evolutionary biology (work on the apicomplexan plastid led to important discoveries about the history of secondary endosymbiosis) and cell biology. Ironically, the apicomplexan plastid is by far the most recently discovered plastid, and yet it is already the best characterised secondary plastid and is becoming a model system in which to study plastid targeting at the molecular level (Waller *et al*., 2000; Foth *et al*., 2003). However, the plastid is also interesting for practical reasons. Plastids, being derived from prokaryotic cyanobacteria, are full of biochemical processes and proteins not found in animals, and are accordingly potentially useful targets for therapeutics. A number of known antibiotics target differences in basic housekeeping activities such as DNA replication, transcription and translation, and several of these have been successfully tested on Apicomplexa (Fichera and Roos, 1997; McFadden and Roos, 1999; Ralph *et al*., 2001). Indeed, some drugs were

known to kill the malaria parasite, but their mode of activity was not understood until the plastid was discovered and the target of the drug found within the plastid. More interesting still, the plastid uses a Type II, or dissociable fatty acid synthase (FAS) while animals use a very dissimilar Type I FAS (Waller *et al*., 1998, 2003). Similarly, the plastid uses the so-called non-mevalonate isopentenyl diphosphate biosynthesis pathway while animals uses the non-homologous mevalonate pathway (Jomaa *et al*., 1999). Both these pathways include a number of enzymes that are the target of well-known drugs that have been successfully tested in Apicomplexa. The plastid can thus be seen as one of the major fundamental differences between the cell biology and biochemistry of these protists and their animal hosts. These differences are the chinks in the armour of any parasite, and should be exploited fully.

5.2. Green Algal Parasites *Helicosporidium* and *Prototheca*

The Helicosporidia are another group of algal parasites, but the story of their cryptic plastid is very different and is just beginning to be told. *Helicosporidium parasiticum* was first described in 1921, but was so unlike any other group of eukaryotes that its evolutionary position was uncertain (Keilin, 1921). The infectious stage of the organism is a cyst containing four cells, three ovoid, internal cells, and a single peripheral cell wound about the others in a helix. *Helicosporidium* is an insect gut parasite and infects when the spore dehisces, or ruptures inside the gut of its host, releasing the helical cell. This cell penetrates a host epithelial cell, and eventually moves through the epithelium to invade the hemolymph, where it develops into a replicating vegetative form (Kellen and Lindegren, 1974; Boucias *et al*., 2001).

Originally, these parasites were classified separately from any other eukaryotic group (Keilin, 1921), but they have also been considered to be fungi, or related to other ''spore-forming'' protists, as were Apicomplexa (Kudo, 1947). Recently, however, the first molecular data from Helicosporidia have shown that none of these affinities is true. Instead, these unique parasites are actually green algae. Phylogenies based on SSU and LSU rRNA, as well as actin and beta-tubulin all show this with strong support (Tartar *et al*., 2002), and the phylogeny of SSU rRNA demonstrates that *Helicosporidium* is a close relative of another algal parasite, *Prototheca*. Unlike *Helicosporidium*, *Prototheca* is predominantly a vertebrate pathogen, and is generally associated with cutaneous infections, although it has been found to infect numerous other tissues (Cho *et al*., 2002; Piyophirapong

et al., 2002; Thiele and Bergmann, 2002). Interestingly, the green algal nature of *Prototheca* was recognised immediately at the time of its original description in 1894, although it was later regarded as a fungus (cited in Butler, 1954; El-Ani, 1967). Although others have argued it to be a fungus since then, there is now an abundance of data, molecular and otherwise, showing *Prototheca* is in fact a close relative of the green algal genus *Chlorella* (Huss and Sogin, 1990). One important exception to this is a misnamed organism, *Prototheca richardsi*, which has been shown to be an icthyosporian, a strange group of parasites closely related to animals and fungi (Baker *et al*., 1999).

Despite its non-photosynthetic, obligately heterotrophic nature, *Prototheca* is known to have retained its plastid, although little is known about its function. The plastid genome of *P. wickerhamii* has been shown to be severely reduced in size (at 54 100 bp), and recently nearly half of this genome was sequenced (Knauf and Hachtel, 2002). As expected, the genome is dominated by genes related to expression, but interestingly a suite of ATPase genes were also found. What the ATPase complex is doing in this organelle is not clear (Knauf and Hachtel, 2002), but it suggests that the role adopted by the plastid in these parasites should be very interesting indeed.

While little is known about the plastid of *Prototheca*, virtually nothing is known about the plastid of the much more highly derived helicosporidia. No organelle matching the description of a plastid has been observed in ultrastructural investigations, but there are now molecular data for a plastid genome. A plastid SSU rRNA has been characterised from *Helicosporidium* (Tartar *et al*., 2003) and has been shown to be related to the plastid SSU from *Prototheca*, as expected. There are no data on the size of the genome, or on what role it might play in the biochemistry of the parasite. In addition, no data are available on nuclear-encoded plastid-targeted proteins in either *Prototheca* or *Helicosporidium*. Such data are potentially very interesting, as they could provide a point of comparison with the apicomplexan plastid and show how two independently derived parasites have re-tooled their plastid as they adapted to parasitism.

5.3. Plastids in Parasitic Plants

Although plants are clearly neither protists nor fungi, and we do not usually consider plants when thinking about pathogens and parasites, there are a number of parasitic angiosperms (Kuijt, 1969) whose plastids provide further interesting points of comparison. Parasitic plants are actually relatively diverse and abundant: at least eleven groups of angiosperms have independently evolved some mode of parasitism, which range considerably

in their degree of host dependence (Nickrent *et al*., 1998). Some of these are still photosynthetic during certain stages of their life cycle, leading one to expect their plastids to more or less resemble those of other plants. In contrast, the most extreme forms of parasitic plants are completely non-photosynthetic, deriving their nutrients and water solely from the invasion of host-plant tissue via a specialised root called a haustorium (Kuijt, 1969). In even these extreme cases, the parasites are clearly derived from photosynthetic plants, and structural evidence for a plastid has been known for some time (Mangenot and Mangenot, 1968; Dodge and Lawes, 1974; Kuijt *et al*., 1985). The angiosperms, therefore, offer a wealth of potential comparative information on the fate of plastids in parasites, but unfortunately very little is known about these organelles.

Most of what we do know about the plastids of these plants comes from the plastid genome. The complete plastid genome has been sequenced for one parasitic angiosperm, *Epifagus virginiana*, or beechdrop (Wolfe *et al*., 1992). The genome is virtually co-linear with that of tobacco, but is severely reduced, almost entirely due to the loss of all genes relating to photosynthesis and photorespiration. Nearly all the remaining genes in the *Epifagus* plastid genome encode proteins involved in transcription and translation of the genome itself. Only four identified genes are not involved in gene expression, and one of these genes encodes the beta subunit of the carboxytransferase component of acetyl-CoA carboxylase, which is involved in fatty acid biosynthesis (Wolfe *et al*., 1992). This gene is at least part of the reason why the plastid genome is retained at all, and indicates that fatty acid biosynthesis is one of the residual functions of the relict plastid, as it is in apicomplexa. Interestingly, the plastid genome of an unrelated parasitic plant, *Cuscuta europeae*, has retained the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), although it has apparently lost CO_2 fixation (Machado and Zetsche, 1990).

It is noteworthy that all four genes in the *Epifagus* plastid genome that are potentially involved in processes other than transcription or translation are known to have been lost in at least one other plastid genome (Downie and Palmer, 1992; Wolfe *et al*., 1992). If a plastid genome encoded only genes involved in its own expression, the genome could be lost. Therefore, it is possible that all functionally important genes have been transferred or lost in some parasitic plant plastids, allowing its genome to be dispensed with altogether. As seen above, this has happened in several instances with mitochondria, but mitochondrial genomes are far more specialised than plastid genomes: the genes they encode are typically restricted to those involved in transcription, translation, and respiration (Gray, 1999). If respiration is lost, the mitochondrial genome could easily be discarded.

In the case of plastids, the genes encoded in plastid genomes of red algae and their derivatives generally represent a far greater diversity of metabolic functions than those found in green algal and plant plastid genomes, which are typically dominated by genes for proteins and RNAs involved in transcription, translation, and photosynthesis (Palmer and Delwiche, 1998). Accordingly, one would predict that green algal or plant plastids would be more likely to dispense with their genomes if photosynthesis were lost (Palmer and Delwiche, 1998). Indeed, there is evidence that parasitic plants have the smallest plastid genomes by far, the smallest known being *Cytinus*, at a mere 20 kb (Nickrent *et al*., 1997b). There are also suggestions based on hybridisation and PCR experiments that some parasitic plants like *Rafflesia* might have lost their plastid genome altogether (Nickrent *et al*., 1997b, 1998). However, this is difficult to distinguish from a high level of divergence, which is seen in the plastid genes of many parasitic plants (Nickrent *et al*., 1997a). If the *Rafflesia* plastid does lack a genome, it is the first plastid known to have taken the process of genome reduction to its conclusion.

6. FUTURE DIRECTIONS

The last decade has seen a complete reversal in the way we regard the fundamental nature of several protist and fungal parasites. In large part, this has come about through phylogenetic analyses, which have given us a better appreciation for the evolutionary history of these parasites and their organelles. Phylogenetics have re-written the history of entire parasitic groups (e.g., the microsporidia are now considered fungi rather than protozoa), their organelles (e.g., hydrogenosomes are now considered to be modified mitochondria: Embley *et al*., 2003), and revealed the presence of organelles that were never expected to exist (e.g., the apicomplexan plastid). As always, however, some of the most exciting scientific discoveries create even more questions than they can answer.

In the case of mitochondria, we are now faced with a growing list of organelles that have been transformed beyond recognition, morphologically and biochemically. The evolutionary histories of many of these are now reasonably well characterised, but their function is, with the exception of hydrogenosomes, poorly understood. The kinds of proteins that have been localised to the various cryptic mitochondria are not altogether informative. In the case of *Entamoeba*, little can be said of function, while in the microsporidia the complete genome of *Encephalitozoon* has allowed some extremely valuable predictions (Katinka *et al*., 2001), but these need to be

verified by localising putative mitochondrial proteins. The same holds for other highly derived mitochondria that have not been discussed, in particular that of the apicomplexan parasite *Cryptosporidium*, where work is just beginning to uncover the unusual biochemistry of this mitochondrion (Riordan *et al*., 1999; Rotte *et al*., 2001). In general, until the complete organellar proteome is determined for each of these cases, it will be difficult to imagine what the cellular role of these organelles may be. In the extreme case of *Giardia*, it has yet to be established if there even is an organelle, or just a few organelle-derived proteins that have been recruited to the cytosol. In this instance, the first priority is obvious: localise the chaperones with homologous antibodies to see whether they can be attributed to a specific cellular compartment. If not, then the mitochondriate nature of *Giardia* will have to be evaluated from the soon-to-be-completed genomic data alone (McArthur *et al*., 2000).

Another aspect of cryptic mitochondria that is emerging as an interesting field is the mechanism by which nuclear-encoded proteins are targeted to the organelle. This system has been worked out for mitochondria, but there are hints that the system used to target proteins to some of these highly derived organelles may be quite different. These hints are vague at present, for example, the possible absence of several translocation proteins in the *Encephalitozoon* genome (Katinka *et al*., 2001) and the lack of obvious leaders on many microsporidian mitochondrial proteins (Fast and Keeling, 2001). In each of these cryptic organelles, this system would have evolved independently starting from a canonical mitochondrial import system, so each case should be looked at individually to see how it might have evolved.

Another important, but frequently overlooked, point to consider is the potential diversity of these organelles within each protist or fungal group. By necessity, we generalise about the function or even presence of an organelle in a group based on evidence from a single genus, for instance *Giardia* representing all diplomonads. In reality, these are large and diverse groups, and we often (as in the case of *Giardia*) focus on some of the strangest members. In most cases our generalisations are probably fairly accurate, but it is possible that we are missing an unexpected diversity of function with some of these unusual organelles, especially given that they have undergone a radical evolutionary change in function at least once in their past. It would be profitable to take a comparative approach to our examination of the cryptic mitochondria by surveying for the presence and function in diverse members of each group where they have been characterised.

Lastly, despite the great successes in identifying cryptic mitochondria in some of the most well-studied amitochondriates, there are still a number of eukaryotes where there remains no evidence for a mitochondrion. One

example of a large hole in our knowledge is the oxymonads, a very poorly studied group of amitochondriates where practically no molecular data are known (Keeling and Leander, 2003). Given the past 10 years of organelle discovery, the safest bet would be that these remaining "amitochondriates" also contain cryptic mitochondria. Nevertheless, it is possible that one or more of these groups may lack the organelle, perhaps even ancestrally (although this is highly doubtful), and it is virtually certain that characterising cryptic mitochondria in each of these lineages will greatly enhance our understanding of how far, and in what directions, the metabolic adaptations of these organelles can extend.

Not surprisingly, the state of our knowledge of cryptic plastids is much different from that of mitochondria, and consequently the major questions are not the same. In the case of the apicomplexan plastid, a flurry of research on plastid function and protein targeting (Waller *et al*., 1998, 2000; Jomaa *et al*., 1999; Sato and Wilson, 2002; Foth *et al*., 2003), helped along by a complete genome sequence (Gardner *et al*., 2002), have quickly addressed many of the main questions in these areas. A clear picture of this plastid's evolutionary history has also made rapid advances, but remains a somewhat woollier issue. The single, ancient origin of a red algal plastid in all chromalveolates has gained considerable support with recent evidence, but needs to be confirmed by additional molecular data. This conclusion would suggest that many non-photosynthetic protists once did, and may still, contain a plastid (Fast *et al*., 2001). These include many parasites such as *Cryptosporidium*, *Perkinsus*, *Blastocystis*, *Pythium*, and *Phytophthora*, as well as all ciliates. There is phylogenetic evidence for one potentially plastid-derived gene from *Phytophthora* (Andersson and Roger, 2002), but no clear-cut evidence for an organelle in any of these organisms, and there are arguments that *Cryptosporidium* lacks a plastid (Zhu *et al*., 2000). On the other end of the spectrum of plastid diversity, the euglenids are known to be closely related to the trypanosomatid parasites (e.g., *Trypanosoma*), and there have been suggestions that the euglenid plastid might also be older than is currently recognised. One intriguing recent analysis of several *Trypanosoma* metabolic enzymes has suggested that they might be derived from an algal endosymbiont (Hannaert *et al*., 2003). Indeed, Cavalier-Smith has proposed that a very large and diverse group of eukaryotes exists, dubbed the Cabozoa, which ancestrally contained a plastid of green algal origin (Cavalier-Smith, 1999, 2000). This group is proposed to encompass euglenids, trypanosomes, diplomonads, Parabasalia, chlorarachniophytes and a number of other lineages. There is presently no direct evidence for this, but the possibility should be confirmed or refuted in coming years as the generation of molecular data from diverse protist groups has accelerated significantly.

Aside from questions about the distribution and evolutionary origin of plastids, there are still many unanswered questions about the function of cryptic plastids in parasites other than Apicomplexa. The Helicosporidia are thought to contain a plastid genome (Tartar *et al*., 2003), but nothing is known about it or the function of the organelle in which it resides. Similarly, little is known about the function of the *Prototheca* plastid, but some intriguing differences between it and that of Apicomplexa have already been identified (Knauf and Hatchel, 2002). An improved understanding of these systems will provide a remarkable opportunity to compare the evolution of a primary, green plastid with a secondary, red plastid in two unrelated groups of intracellular parasites that have inherited plastids by very different evolutionary routes. Even less is known about the function of plastids in most parasitic plants, and the genomes of these plastids are intriguing as they appear to be the most reduced of all plastid genomes, perhaps even having been eliminated altogether (Nickrent *et al*., 1997b, 1998). In addition, there are a great number of algae that have lost photosynthesis for reasons other than adapting to parasitism. Most of these have simply become heterotrophs or osmotrophs, and it would be interesting to compare and contrast how their plastids have evolved with those of parasitic algae to see if any significant trends can be identified. The complete plastid genome of an osmotrophic euglenid, *Astasia longa*, has now been sequenced (Gockel and Hachtel, 2000). Like plastids in parasitic algae, its genome is highly reduced (about one-half the size of the plastid genome of the closely related *Euglena gracilis*) and virtually all of the remaining genes encode proteins involved in gene expression. Like parasites, the genome also contains a small number of genes related to other functions (in the case of *Astasia*, the gene for the Rubisco large subunit). Whether the plastid genomes of non-photosynthetic heterotrophs and osmotrophs like *Astasia* tend to retain different kinds of genes than those of parasites is presently unclear, but comparing these genomes could provide some interesting glimpses into the different ways that the plastid can adapt to the loss of photosynthesis.

Lastly, there are a number of other organelles of interesting or questionable origin in a diverse variety of protist parasites. For instance, the kinetoplastid glycosome is a membrane-bounded compartment for glycolysis and purine salvage (Parsons *et al*., 2001) that is restricted to this group: in other eukaryotes these processes take place in the cytosol (although glycolytic enzymes have been found to be targeted to the mitochondria in some heterokonts: Liaud *et al*., 2000). Glycosomes appear to have evolved from peroxisomes (Parsons *et al*., 2001; Hannaert *et al*., 2003), and exactly how they acquired their current metabolic pathways is an intriguing question. Several other organisms have cryptic organelles that

look much like hydrogenosomes, but have not been characterised biochemically, for instance *Psalteriomonas*, *Postgaardi*, *Trimastix*, and *Carpediemonas* (Broers *et al*., 1990; Simpson *et al*., 1997, 2002; O'Kelly *et al*., 1999; Simpson and Patterson, 1999). Characterising these organelles could show that the distribution of hydrogenosomes is even broader than initially imagined, or could reveal a new kind of cryptic organelle with metabolic functions different once again from those we now know. Considering only those facts that we already have, it is clear that the evolutionary trajectory of these unusual organelles can take many unpredictable paths. The metabolism that has been described consists of an odd mix of ancestral functions cobbled together with new enzymes from various sources, reflecting the action of evolution: building new machines with the pieces at hand, in an *ad hoc* fashion, and as the need arises.

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NOTE

A recent report (Tovar, J., Leon-Avila, G., Sánchez, L.B., Sutak, R., Tachezy, J., van der Giezen, M., Hernández, M., Müller, M. and Lucocq, J.M. 2003. Mitochondrial remnant organelles of *Giardia* function in iron-sulphur cluster metabolism. Nature, in press) has now provided unambiguous evidence for a relict mitochondrion in the diplomonad parasite, *Giardia*. This represents one of the last major groups of amitochondriate eukaryotes and further supports the conclusion that no known eukaryotes are ancestrally amitochondriate (Oxymonads are one group where no evidence is yet available, but they have not been thoroughly examined).

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