Mitochondrial Type Iron-Sulfur Cluster Assembly in the Amitochondriate Eukaryotes *Trichomonas vaginalis* and *Giardia intestinalis*, as Indicated by the Phylogeny of IscS

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Pyridoxal-5′-phosphate-dependent cysteine desulfurase (IscS) is an essential enzyme in the assembly of FeS clusters in bacteria as well as in the mitochondria of eukaryotes. Although FeS proteins are particularly important for the energy metabolism of amitochondrial anaerobic eukaryotes, there is no information about FeS cluster formation in these organisms. We identified and sequenced two IscS homologs of *Trichomonas vaginalis* (TviscS-1 and TviscS-2) and one of *Giardia intestinalis* (GiiscS). TviscS-1, TviscS-2, and GiiscS possess the typical conserved regions implicated in cysteine desulfurase activity. N-termini of TviscS-1 and TviscS-2 possess eight amino acid extensions, which resemble the N-terminal presequences that target proteins to hydrogenosomes in trichomonads. No presequence was evident in GiiscS from *Giardia*, an organism that apparently lacks hydrogenosmes or mitochondria. Phylogenetic analysis showed a close relationship among all eukaryotic IscS genes including those of amitochondriates. IscS of proteobacteria formed a sister group to the eukaryotic clade, suggesting that *isc*-related genes were present in the proteobacterial endosymbiotic ancestor of mitochondria and hydrogenosomes. NifS genes of nitrogen-fixing bacteria, which are IscS homologs required for specific formation of FeS clusters in nitrogenase, formed a more distant group. The phylogeny indicates the presence of a common mechanism for FeS cluster formation in mitochondriates as well as in amitochondriate eukaryotes. Furthermore, the analyses support a common origin of *Trichomonas* hydrogenosomes and mitochondria, as well as secondary loss of mitochondrion/hydrogenosome-like organelles in *Giardia*.

Introduction

Amitochondriate eukaryotes can be divided into two metabolic types (Martin and Müller 1998; Müller 1998). Type I organisms such as *Giardia* and *Entamoeba* lack organelles involved in core energy metabolism, while type II organisms (trichomonads, some ciliates, and chytrid fungi) harbor a double-membrane limited organelle, the hydrogenosome (Müller 1993; Hackstein et al. 1999; Kulda 1999). The hydrogenosome is the site of the FeS protein-mediated metabolism of pyruvate and the formation of molecular hydrogen, which is accompanied by substrate-level phosphorylation ATP synthesis. In type I amitochondriates, the FeS protein-dependent pyruvate metabolism takes place in the cytosol (Reeves 1984; Ellis et al. 1993). The evolutionary origin of amitochondriate eukaryotes is much debated. Although they were long regarded as ancestral premitochondrial lineages (Cavalier-Smith 1987), recent evidence suggests that they had experienced the endosymbiotic event leading to the establishment of the mitochondrion (Embley and Hirt 1998; Martin and Müller 1998; Roger 1999). In type II organisms, hydrogenosomes are regarded as descendants of a common endosymbiont that evolved to either mitochondria or hydrogenosomes (Martin and Müller 1998; Müller 1997). A common origin of the two organelles is supported by a number of similarities in their structure, function, and biogenesis (Johnson, Lahti, and Bradley 1993; Benchimol, Johnson, and De Souza 1996; Bui, Bradley, and Johnson 1996; Bradley et al. 1997; Dyall et al. 2000), as well as by phylogenetic analysis of several hydrogenosomal metabolic enzymes (Länge, Rozario, and Müller 1994; Hrdý and Müller 1995*a*, 1995*b*) and heat shock proteins (Müller 1997; Embley and Hirt 1998). Although neither mitochondria nor hydrogenosomes have been found in type I organisms, genes of probable mitochondrial origin have been identified in *Giardia* (Roger et al. 1998) and *Entamoeba* (Clark and Roger 1995). Moreover, a putative mitochondrial ''remnant,'' the mitosome (Tovar, Fischer, and Clark 1999) or crypton (Mai et al. 1999), has recently been detected in *Entamoeba* (Müller 2000). These considerations led us to the hypothesis that FeS proteins operating in the energy metabolism of mitochondrial as well as secondarily amitochondrial organisms were present in the common ancestral organelle. If so, a common mechanism of FeS cluster assembly may operate in amitochondriate and in mitochondrial eukaryotes.

In spite of the importance of FeS proteins for all living cells, little is known of how and where FeS clusters are synthesized in vivo and which proteins are involved in their insertion into the apoproteins. The best-characterized enzyme participating in this process is a pyridoxal-5′-phosphate-dependent cysteine desulfurase which catalyzes the formation of L-alanine and elemental sulfur by using L-cysteine as substrate. Initially, the enzyme was described as NifS in *Azotobacter vinelandii* (Zheng et al. 1993), in which it provides sulfur for FeS cluster formation in nitrogenase (Zheng and Dean 1994). Later, a NifS homolog designated IscS (iron-sulfur cluster) was found in *A. vinelandii*, as well as in a number

Abbreviations: ML, maximum likelihood; MP, maximum parsimony; NJ, neighbor-joining; PLP, pyridoxal-5′-phosphate.

Key words: IscS, iron-sulfur cluster, hydrogenosome, *Trichomonas vaginalis*, *Giardia intestinalis.*

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of non–nitrogen-fixing bacteria. It has been proposed that IscS plays a general role in the formation of FeS clusters or repair of FeS proteins with a housekeeping function (Zheng et al. 1998). More recently, a major role of IscS in de novo FeS cluster synthesis has been demonstrated using an *iscS* deletion strain of *Escherichia coli* (Schwartz et al. 2000). Importantly, IscS homologs have been identified in the genomes of diverse eukaryotes (*Arabidopsis, Caenorhabditis, Drosophila, Homo, Mus, Saccharomyces*), suggesting a general role for IscS in FeS cluster formation. In mice (Nakai et al. 1998) and yeast (Strain et al. 1998), IscS is localized in mitochondria. In human cells, the IscS homologs are targeted either to mitochondria or to the cytosol and nucleus (Land and Rouault 1998). Mutation in an *iscS*-like gene in yeast (*NFS1*) caused reduction in the activities of the mitochondrial FeS proteins, aconitase and succinate dehydrogenase (Strain et al. 1998). According to Kispal et al. (1999) and Lill and Kispal (2000), mitochondria also play a crucial role in the FeS cluster formation of extramitochondrial FeS proteins. In addition, IscS homologs have been found to mediate several other functions that are independent of FeS cluster assembly but require IscS as a sulfur donor. Thus, IscS homologs are involved in biosynthesis of thiamin (Lauhon and Kambampati 2000), NAD (Sun and Setlow 1993), 4-thiouridine (Kambampati and Lauhon 1999), and molybdopterin (Amrani et al. 2000). Finally, IscS/NifS homologs mediate release of elemental selenium from L-selenocysteine (Mihara et al. 2000), and they may participate in tRNA splicing (Kolman and Söll 1993).

IscS has not been reported in amitochondriate eukaryotes, although FeS proteins are of particular importance for these organisms (Müller 1998). Here we report the identification of genes encoding IscS in the type II organism *Trichomonas vaginalis* and the type I organism *Giardia intestinalis.* Phylogenetic analysis indicates the presence of a common mechanism for FeS cluster formation in mitochondria and hydrogenosomes, as well as in organisms that secondarily lost the mitochondrion/hydrogenosome-like organelles.

Materials and Methods

Organisms and Genomic DNA Preparation

Trichomonas vaginalis strain NIH-C1 (ATCC 30001) and *G. intestinalis* strain WB, clone 6 (ATCC 300957), were used. Genomic DNA was isolated from *T. vaginalis* using a guanidium thiocyanate procedure (Wang and Wang 1985) and from *G. intestinalis* using a Blood & Culture DNA kit (Qiagen, Chatsworth, Calif.).

Probe Preparation, Cloning, and Screening of the Genomic Library

To obtain probes for screening a *T. vaginalis* genomic library, two pairs of degenerate primers, a GC-rich one and an AT-rich one, were designed based on the conserved regions of IscS/NifS sequences in GenBank (National Center for Biomedical Information): EIIFTSGATE (GC-rich sense: 5′-GARATYATYTTCTCVTCHGGHGCHACHGAR-3′; AT-rich sense: 5′-GAAATWATWTTYACWWSWGGWGCWACWGAA) and HKIH/YGPKGV/IG (GC-rich antisense: 5′-CCRAYDCCYTTTGGDCCRTRRATYTTRTG-3′; AT-rich antisense: 5′-CCWAYWCCYTTTGGWCCRTRWATYTTRTG-3′). Corresponding fragments were amplified by PCR, purified with a gel extraction kit (Qiagen) and cloned into pCR 2.1 vector (TA cloning kit, Invitrogen). The inserts were excised from the vector, gel-purified, and labeled by means of a Random Primers DNA Labeling System (GIBCO/BRL) with α-[^{32}P]dATP. These probes were used for screening a genomic DNA library in λ ZAP II vector (Stratagene). The sequences of positive clones were determined for both strands by primer walking.

Nucleotide sequences of *Escherichia coli* and *Saccharomyces cerevisiae IscS*/*NifS* homolog genes were used to search the *Giardia lamblia* genome sequence database (http://www.mbl.edu/baypaul/Giardia-HTML/index2.html; McArthur et al. 2000) with the BLAST program. Clones Ai0824 and Ki1686 contained sequences homologous to the N- and C-terminal ends of the bacterial and eukaryotic homologs. Based on the nucleotide sequence of these clones, we designed a pair of oligonuclueotide primers (sense: 5′-GATGACGAGCGTGCAAGGAAAGCTC-3′; antisense: 5′-GGTGACTACATGCGGATGCTCAGCC-3′) located in the 5′ and 3′ untranslated regions of the putative *G. intestinalis nifS* homolog gene, respectively. PCR reactions, utilizing these oligonucleotides and *G. intestinalis* genomic DNA as template, amplified a 2.4-kb fragment that was purified, cloned into the pCR2.1 vector (Invitrogen), and sequenced.

Sequence Alignment

Nucleotide and protein database searches were performed at the National Center for Biomedical Information using the BLAST program (Altschul et al. 1997). Sequences were extracted from databases using the BlastAli program (http://www.joern-lewin.de/). The IscS sequences of *T. vaginalis* and *G. intestinalis* were aligned to sequences from 64 taxa using ClustalX (Thompson et al. 2000). The alignment was further edited visually with the use of the ED program of MUST (Philippe 1993). The alignment of all 67 taxa resulted in 231 shared amino acid positions, while an alignment of 21 selected taxa consisted of 362 shared amino acid positions. The *T. vaginalis TviscS-1, T. vaginalis TviscS-2,* and *G. intestinalis GiiscS* sequences have been submitted to GenBank under accession numbers AF321005, AF321006, and AF311744, respectively.

Phylogenetic Analysis

Phylogenetic relationships were analyzed by means of the Neighbor-Joining (NJ) and Maximum-Parsimony (MP) methods using PHYLIP, version 3.6 (Felsenstein 1989), and by the Maximum-Likelihood (ML) method using the PROTML program in MOLPHY, version 2.3 (Adachi and Hasegawa 1996). The ML tree was constructed by local rearrangement of an NJ tree using the Jones-Taylor-Thornton model of amino acid substitutions with the F-option (JTT-F) to account for amino

acid frequencies in the data set. User-defined trees were analyzed to compare alternative topologies (Kishino and Hasegawa 1989). The local bootstrap proportion value was calculated for each internal branch of the ML tree using a local rearrangement option of the PROTML program. Bootstrap support for distance and parsimony analyses were based on 100 resampled data sets using SEQBOOT, PHYLIP, version 3.6.

Results and Discussion

Analysis of *T. vaginalis* and *G. intestinalis iscS* Genes and Putative Translation Products

Sequences for two complete *iscS* genes from *T. vaginalis* (*TviscS-1* and *TviscS-2*) and for one gene from *G. intestinalis* (*GiiscS*) were obtained. The *T. vaginalis* genomic DNA library was screened with two probes derived from PCR products. The products were amplified using the A+T-rich or the G+C-rich degenerate primer pairs. Each pair amplified a distinct DNA fragment of about 4.4 kb. The two products displayed only 60% nucleotide sequence identity. Both fragments were identified as IscS/NifS homologs by a BLAST search. These products used as probes recognized separate sets of positive clones of the genomic library containing either *TviscS-1* or *TviscS-2* genes. No cross-reactivity between these clones was observed. The isolated clones contained complete putative open reading frames (ORFs) without intron-like sequences. The G+C content of *TviscS-1* was rather low (39.7%), and that of *TviscS-2* was higher (46.7%). The *TviscS-1* and *TviscS-2* coded for proteins 385 and 411 amino acids in length, respectively. Predicted molecular mass and an isoelectric point for the putative products for TviscS-1 were 41.9 and 8.2, while they were 44.8 and 8.2 for TviscS-2, respectively. The *G. intestinalis* gene isolated contained a 52% G+C-rich ORF coding for a polypeptide of 433 amino acids with a predicted molecular mass of 47.7 and an isoelectric point of 6.3.

Amino acid sequences deduced from the *Trichomonas* and *Giardia* genes were compared with IscS/NifS homologs from 64 species, including bacteria, fungi, plants, invertebrates, and vertebrates (alignment available on request from J.T.). An alignment of selected sequences including that of eubacterial IscS from *A. vinelandii* and mitochondrial sequence from *S. cerevisiae* is shown in figure 1. Both *Trichomonas* and *Giardia* sequences contained all conserved regions proposed to mediate the cysteine desulfurase activity in IscS/NifS-like proteins: (1) His111 (numbered according to TviscS-1), which is involved in initial deprotonation of the substrate (Kaiser et al. 2000); (2) the pyridoxal-5′-phosphate-binding site with the Schiff base forming Lys222 residue and Asp187 and Gln190, which bind the pyridine nitrogen and the phenolate oxygen of PLP, respectively, and residues involved in forming an additional six hydrogen bonds anchoring the phosphate group: Thr82, His221, Ser/Thr219, and Thr250 (Zheng et al. 1993); and (3) the substrate-binding site including Cys371, which provides a reactive cysteinyl residue (Zheng et al. 1994), as well as Arg362, Asn162, and Asn 41, which anchor the cysteine with a salt bridge and hydrogen bond (Kaiser et al. 2000).

Importantly, the alignment revealed short N-terminal extensions of the trichomonad IscS sequences which were not present at the N-terminus of IscS in *Giardia* or in the eubacterial sequences. The PSORT II program (http://psort.nibb.ac.jp/), designed for prediction of cleavage sites for mitochondrial presequences, recognized the SRS/YF motif with a characteristic arginine at position −2 relative to the cleavage site at the TviscS-2 N-terminus (fig. 2). The TviscS-2 extension was typically serine-rich, consisting of eight amino acids, which started with leucine. It resembled N-terminal leader sequences found in proteins targeted to the *Trichomonas* hydrogenosome and can be assumed to have the same function (fig. 2). Interestingly, TviscS-1 also started at the same position and contained an eight-amino-acid N-terminal extension; however, it did not contain the typical −2 arginine, and PSORT II did not recognize the consensus cleavage sequence. Nevertheless, a function of TviscS-1 presequence in organelle targeting cannot be ruled out, as a presequence without arginine has recently been reported for the hydrogenosomal membrane protein Hmp31 (Dyall et al. 2000). No N-terminal extension of IscS was expected in *Giardia,* as this organism contains neither mitochondria nor hydrogenosomes. Lack of the N-terminal extension was previously reported in *Giardia* Hsp60, another mitochondrial-type protein recognized in this organism (Roger 1998).

The presence of all key elements required for cysteine desulfurase activity in the deduced proteins suggests that IscS homologs are involved in Fe-S cluster assembly in *T. vaginalis* and *G. intestinalis.* The presence of the conserved N-terminal leader sequence in TviscS-2 suggests that in trichomonads the IscS-like proteins are targeted to hydrogenosomes, which are the likely sites of Fe-S cluster assembly. In *Giardia,* the FeS clusters are possibly assembled in cytosol.

A C-terminal sequence signature differentiates proteobacterial and eukaryotic IscS from homologs in all other organisms. TviscS-2 and GiiscS also contain this signature, which consists of 20 to 21 amino acids with consensus sequence SPL(W/Y)(E/D)(M/L)X(K/Q)XG(I/V)D(L/I)XX(I/V)XWXXX (fig. 1). NifS genes of nitrogen-fixing bacteria also possess a similar C-terminal extension that starts with the Ser-Pro motif, but the subsequent sequence is not conserved. The only sequence from a eukaryote that lacks this signature is TviscS-1. The lack of this signature and the ''atypical'' N-terminal extension might indicate that *Tvisc-1* is a pseudogene or that its product has a different function or localization. Eukaryotic IscS is distinguished from all other organisms, including proteobacteria, by the invariable Cys113 in the substrate deprotonation region. This residue is present in both trichomonad *iscS* products as well as in GiiscS. Prokaryotes possess Ala, Ser, or Gly at this position. Interestingly, *Giardia* IscS possessed two unique highly hydrophilic inserts, Thr137-Glu145 and Glu300-Ser321, which are not present in any of the 66 other species. It will be of interest to determine the function

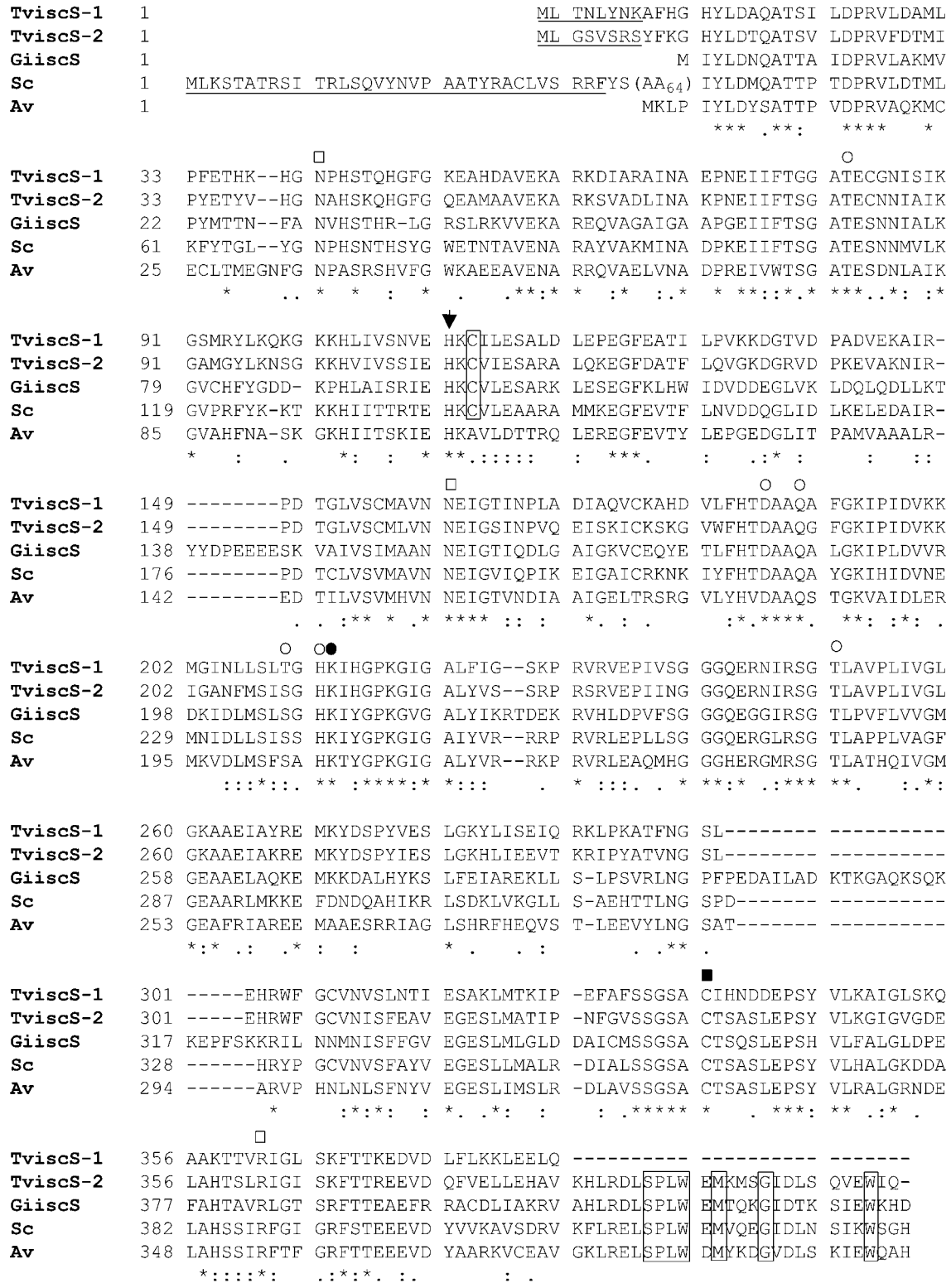


FIG. 1.—Sequence alignment of putative *Trichomonas vaginalis* (TviscS-1, TviscS-2) and *Giardia intestinalis* (GiiscS) IscS proteins with eubacterial (*Azotobacter vinelandii,* Av) and mitochondrial (*Saccharomyces cerevisiae,* Sc) homologs. Conserved lysine and other residues involved in PLP binding are indicated by closed (●) and open circles (○), respectively. Invariable cysteine is highlighted by a closed square (■), while other residues involved in substrate binding are indicated by open squares (□). An arrow indicates the conserved histidine involved in the substrate deprotonation. Putative N-terminal presequences are underlined. Cystein signatures of eukaryotic IscS and C-terminal conserved residues typical for eukaryotic/eubacterial IscS are boxed. (''*'' indicates fully conserved residue, '':'' indicates conserved ''strong'' groups, and ''.'' indicates conserved ''weaker'' groups according to ClustalX). For accession numbers see figure 3.

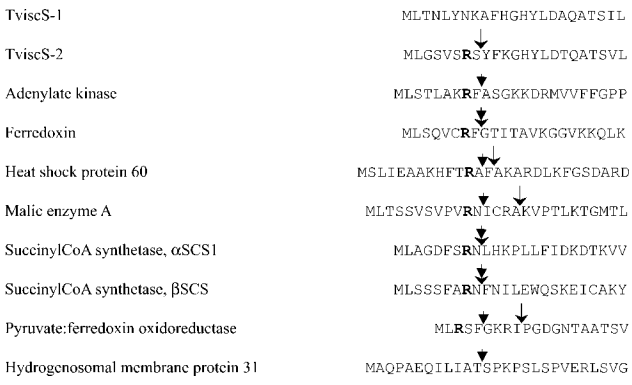


FIG. 2.—Comparison of N-terminal amino acid sequences of TviscS-1 and TviscS-2 with leader sequences known to target hydrogenosomal proteins to the organelle. The cleavage sites recognized by PSORT program (http://psort.nibb.c.jp/) are indicated by thin arrows, and those determined by N-terminal sequencing (Dyall et al. 2000) are indicated by thick arrows. Accession numbers of compared genes are as follows: adenylate kinase, P49983; ferredoxin, P21149; heat shock protein 60, Q95058; malic enzyme A, AAA92714; succinylCoA synthase αSCS1, P53399; succinylCoA synthase βSCS, Q03184; pyruvate : ferredoxin oxidoreductase A, AAA85494; hydrogenosomal membrane protein Hmp31, AF216971.

of such inserts, which might be associated with a specific localization of the gene product in *Giardia*.

Our sequencing data provide a solid basis for the prediction of the function of the products of *iscS* genes in amitochondriate eukaryotes. However, further studies are required to confirm their physiological function and cell localization.

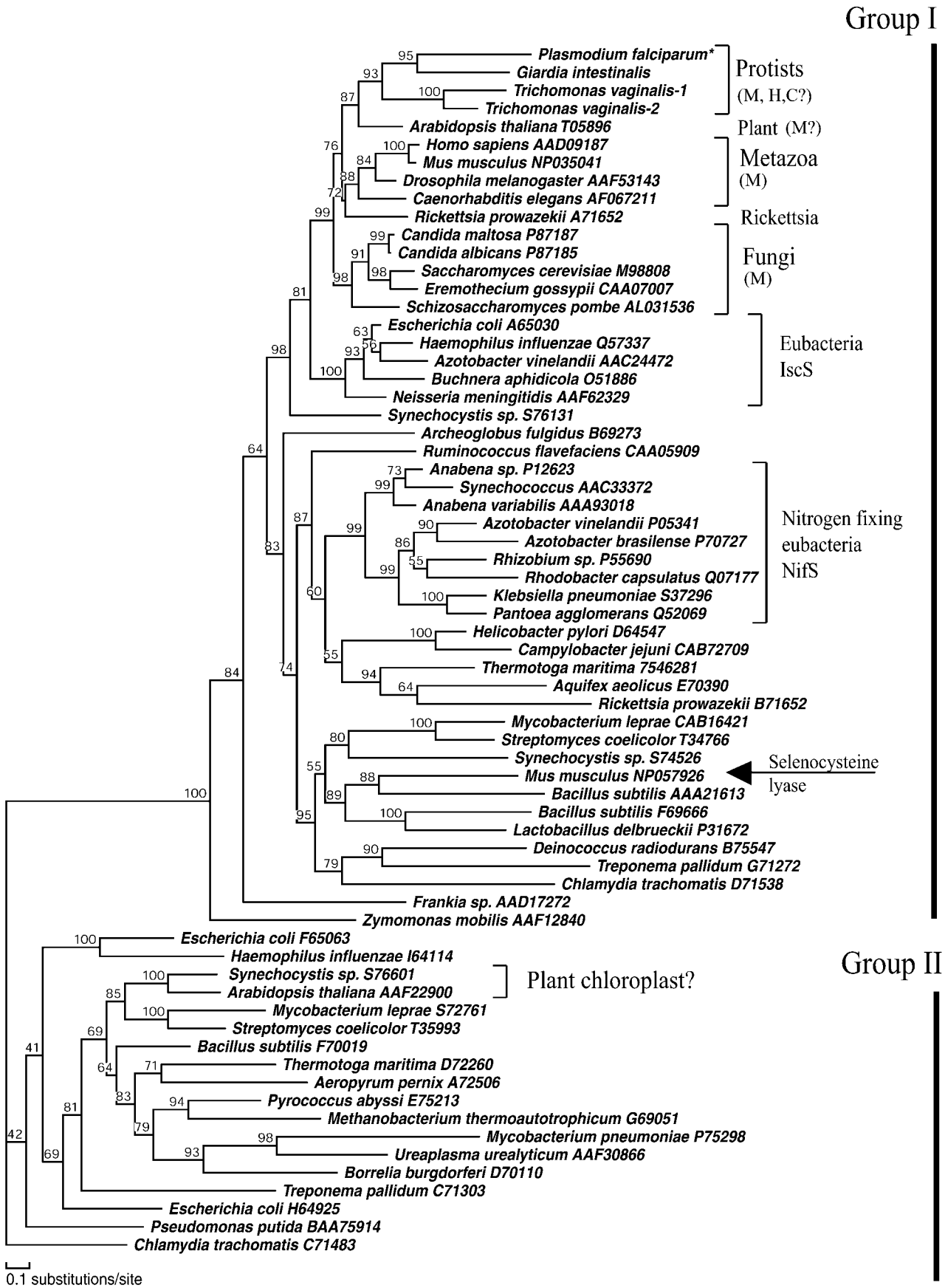
Phylogenetic Analysis of IscS/NifS-like Sequences

In all global phylogenetic reconstructions, IscS/NifS-like homologs formed two distinct groups that were previously designated groups I and II (Mihara et al. 1997). The IscS sequences of *Trichomonas* and *Giardia* and those of the mitochondrial homologs in other eukaryotes formed a single clade (group I) with a high bootstrap value (99%) using the local rearrangement option of the PROTML program (fig. 3). Within this clade, *Trichomonas* and *Giardia* formed a subgroup together with *Plasmodium falciparum* and *Arabidopsis thaliana*. The second eukaryotic subgroup consisted of metazoan IscS, and the third group comprised homologs in fungal mitochondria. The α-proteobacterium *Rickettsia prowazeki*, often considered a close relative to the mitochondrial ancestor, clustered together with metazoan mitochondrial IscS (Andersson and Kurland 1999).

The IscS of proteobacteria formed a sister group to eukaryotes. It is known that nitrogen-fixing bacteria possess two separate gene clusters that are involved in FeS cluster assembly: products of the *nif* gene cluster, including NifS, participate in nitrogenase-specific FeS cluster formation (Zheng and Dean 1994), and *isc* gene products, including IscS, which have a more general function in the assembly of other Fe-S proteins (Zheng et al. 1998). Our analysis suggests that *isc*-related genes were present in the proteobacterial endosymbiotic ancestor of mitochondria and hydrogenosomes. The more specialized NifS sequences formed a distinct group with 99% local bootstrap support in the PROTML analysis which contained exclusively nitrogen-fixing proteobacteria.

Additional IscS/NifS-like homologs of eukaryotic organisms (*Mus musculus* and *A. thaliana*) also appeared in another two distinct clades. The mouse homolog was located in a heterogeneous eubacterial group (fig. 3). This gene codes for the cytosolic pyridoxal-5′-phosphate-dependent selenocysteine lyase, which resembles NifS in primary structure as well as in catalytic function (Mihara et al. 2000). If the partial sequence of a human counterpart was also included in the analysis (data not shown), it was a sister group to the mouse sequence. The function of bacterial gene products of this clade has not been studied except in *Synechocystis* sp. (S74526 corresponds to sll0704 in CyanoBase http://www.kauza.or.jp/cyano/ investigated by Kato et al. [2000]) and *Bacillus subtilis* (AAA21613 corresponds to the *nifS*-like gene according to Sun and Setlow [1993]). The product of the *Synechocystis* gene showed selenocysteine lyase activity, although it also acted on L-cysteine sulfinic acid and other substrates (Kato et al. 2000). The *B. subtilis* gene product has been suggested to participate in NAD biosynthesis (Sun and Setlow 1993). Thus, it is likely that other members of this clade also have biochemical functions that are different from those of NifS and IscS proteins. The topology of *A. thaliana* genes was of particular interest. While one gene was related to the subtree of genes coding for mitochondrial IscS in protists, a second gene was placed in group II together with *Synechocystis* S76601. Since cyanobacteria share a common ancestor with plastids, we analyzed the second *A. thaliana* sequence for its possible subcellular localization with the PSORT program. The analysis gave the highest score for a chloroplast stroma localization (certainty = 0.501) of the gene product. This analysis suggests that the second IscS homolog of *A. thaliana* may operate in the chloroplast. However, it is difficult to predict its possible function. Group II consists of the most divergent NifS/IscS homologs. Function has been established only for products of two *E. coli* genes, which encode cysteine sulfinate desulfinase (F65063; Mihara et al. 1997), and for selenocysteine lyase (H64925; Fujii et al. 2000). Thus, the *A. thaliana* IscS homolog, as well as other members of this heterogenous bacterial group, might have functions different from FeS cluster formation, and their distance from genes of group I might reflect different evolutionary pressures. In any case, further biochemical studies on the functions of group II members are required.

The global gene tree showed that amitochondrial and mitochondrial IscSs share a common eubacterial ancestor, suggesting a common biosynthetic mechanism for FeS proteins. The tree also showed that both eukaryotes and bacteria possess several paralogous or orthologous IscS/NifS-like genes. The topology of these gene trees possibly reflects their specialized function or cell localization more than their large-scale phylogenetic relationships. Therefore, in a subsequent analysis we restricted the data set to eukaryotic and proteobacterial IscSs. The trees constructed by the ML, MP, and NJ



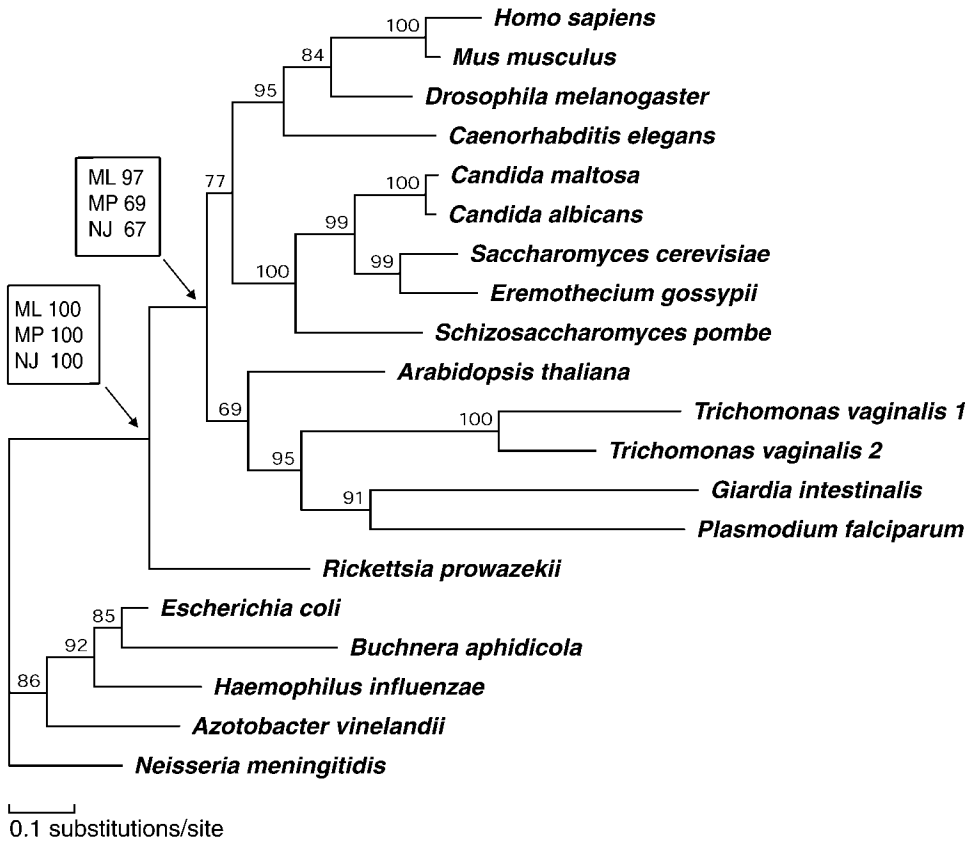


FIG. 4.—Tree of eukaryotic-proteobacterial IscS constructed with the Maximum-Likelihood (ML) method (Jones-Taylor-Thornton model). Bootstrap values (local bootstrap proportion) of the PROTML analysis are shown at the considered nodes. For the node of the main interest, the bootstrap values calculated by the ML, Maximum Parsimony (MP), and Neighbor-Joining (NJ) methods are given in a box.

methods confirmed the close relationship between amitochondrial and mitochondrial IscS with high bootstrap support (fig. 4). The robustness of the relationship within the eukaryotic group was further assessed through the analysis of alternative tree topologies. We defined six branches on the tree: (1) amitochondriates and *Plasmodium*, (2) *Arabidopsis*, (3) Fungi, (4) Metazoa, (5) *Rickettsia*, and (6) Proteobacteria. Evaluation of the 105 alternative trees confirmed a common ancestry of genes from amitochondriate and mitochondriate organisms. However, the branching order within the eukaryotic clade was not resolved, as several alternative positions for eukaryotic subtrees with comparable significance were found (table 1). We further suspected that the subtree of protists could be affected by long-branch attraction in spite of high bootstrap support. Thus, the positions of the *Trichomonas* and *Giardia* genes were tested using a data set from which we removed the most divergent sequences of *A. thaliana* and *P. falciparum*. The analysis clearly showed the instability of the protist group. Although NJ and MP reconstruction placed *Trichomonas* and *Giardia* as sister taxa with bootstrap support of 85% and 87%, respectively, ML constraint analysis gave comparable support to several alternative hypotheses. In the best tree topology and in four other tree topologies with $\Delta \ln L < 1$ SE from the best tree, *Trichomonas* and *Giardia* were not placed as sister taxa (table 1). Nevertheless, in all alternative trees, both amitochondriates were part of the eukaryotic clade.

Our results indicate that a common mechanism mediates FeS cluster assembly in mitochondriate and amitochondriate eukaryotes, even though different sets of FeS proteins function in these organisms (Müller 1998). Both mitochondriate and amitochondriate eukaryotes require FeS proteins for single-electron transport processes associated with ATP production linked to the utilization of oxygen (mitochondriates) or not (type I and II amitochondriates). In mitochondria, FeS proteins are involved in the respiratory chain (subunits of complexes I, II, and III) and in the citric acid cycle (aconitase), while low–redox-potential FeS proteins such as pyruvate : ferredoxin oxidoreductase and ferredoxin are pre-

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FIG. 3.—Phylogenetic tree based on analysis of IscS/NifS amino acid sequences. The tree was constructed using the maximum-likelihood method with the Jones-Taylor-Thornton model of amino acid substitution (JTT-F). Local bootstrap values calculated by the PROTML program are attached to the internal nodes. The cell localization is indicated by M (mitochondria), H (hydrogenosomes), and C (cytosol). * These sequence data were produced by the *Plasmodium falciparum* Sequencing Group at the Sanger Centre and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/malaria2/unfinished_ORFS/orf_dna_sequences.

Table 1
Maximum-Likelihood Analysis of Alternative Tree Topologies Using the JTT-F Model of Amino Acid Substitution

Tree Topology	ln *L*	Δln *L*	SE	RELL-BP
Significance of tree topology within the eukaryotic–proteobacterial clade				
(M, F, ((A, **Prot**), (R, P)))	−8,458.1	0.0	BEST	0.3596
(M, (F, **Prot**), (A, (R, P)))	−8,458.5	−0.4	10.5	0.3347
(M, F, (**Prot**, (A, (R, P))))	−8,461.0	−2.9	7.6	0.0575
(M, (F, **Prot**), (R, (A, P)))	−8,467.9	−9.9	13.8	0.0476
(M, F, (**Prot**, (R, (A, P))))	−8,469.1	−11.1	12.1	0.0262
(M, **Prot**, (F, (A, (R, P))))	−8,464.3	−6.3	8.9	0.0012
Alternative tree topology of *Trichomonas* and *Giardia* within the eukaryotic–proteobacterial clade with *Plasmodium* and *Arabidopsis* removed from the data set				
(M, F, **Trich**), R, (**Gia**, E)))	−7,739.3	0.0	BEST	0.3731
(M, (F, **Trich**), (P, (R, **Gia**)))	−7,744.3	−5.1	7.4	0.0833
(M, F, ((**Gia, Trich**), (R, P)))	−7,745.8	−6.5	12.8	0.0728
(M, F, (**Trich,** (R, (**Gia**, P))))	−7,743.1	−3.8	4.6	0.0655
(M, **Trich**, (F, (R, (**Gia**, P))))	−7,743.5	−4.2	4.6	0.0541
(M, (**Gia, Trich**), (F, (R, P)))	−7,746.6	−7.3	12.9	0.0393
(M, **Trich**, (F, (P, (**Gia**, R))))	−7,747.5	−8.2	8.7	0.0233

NOTE.—Tree topologies with Δln $L < 1$ SE are shown. ln *L* = log likelihood; Δln *L* = log likelihood difference of the tree from that of the best tree; SE = standard error of Δln *L* (Kishino and Hasegawa 1989); RELL–BP-bootstrap probabilities estimated by the RELL method among 105 alternatives (Kishino, Miyata, and Hasegawa 1990); A = *Arabidopsis*; F = fungi; Gia = *Giardia*; M = Metazoa; Prot = protists; P = proteobacteria; R = *Rickettsia*; Trich = *Trichomonas.*

sent in the hydrogenosomes of type I and in the cytosol of type II amitochondriates. Hydrogenosomes also contain hydrogenase. The presence of a common machinery responsible for FeS cluster formation and incorporation into ''aerobic'' and ''ananerobic'' types of apoproteins is supported by (1) the identification of the genes encoding IscS, a key member of machinery involved in FeS cluster assembly, in *T. vaginalis* and *G. intestinalis*, and (2) the placement of IscS of these amitochondrial organisms within the eukaryotic clade in phylogenetic analysis. In addition, a partial sequence of IscU, another protein involved in FeS cluster formation, has been identified in the database of the *Giardia* genome project (http://www.mbl.edu/baypaul/Giardia-HTML/index2.html).

A common origin of FeS cluster formation in mitochondriate and amitochondriate eukaryotes could be explained by the recently proposed hydrogen hypothesis of eukaryotic origin (Martin and Müller 1998). The hypothesis assumes that all eukaryotes, including contemporary amitochondrial organisms, once harbored the mitochondrion/hydrogenosome-like organelle derived from a proteobacterial endosymbiont. The ancestral endosymbiont is viewed as a facultatively anaerobic proteobacterium which possessed both anaerobic and aerobic metabolic machineries for electron transport–linked ATP production, including ''aerobic'' and ''anaerobic'' types of FeS proteins. A possible scenario is that the ''anaerobic'' set of FeS proteins was preserved in hydogenosomes, whereas the ''aerobic'' set was preserved in mitochondria. Both organelles inherited the common mechanism of the FeS cluster assembly. This scenario is supported by the close phylogenetic relationship between eukaryotic and proteobacterial IscS proteins. Our hypothesis is also congruent with a common origin of mitochondria and *Trichomonas* hydrogenosomes, as well as a secondary loss of the mitochondrion/hydrogenosome-like organelle in *Giardia.* We cannot, however, rule out alternative explanations for the origin of hydrogenosomes and biochemistry of amitochondrial eukaryotes, including the mechanism of FeS cluster assembly. Indeed, independent lateral gene transfers (Doolittle 1998) or preservation of certain biochemical pathways from an anaerobic past of eukaryotic evolution might be involved. Nevertheless, comparative analysis of mechanisms responsible for the formation of FeS clusters, which are considered to be among the most ancient biologically active metal cofactors (Cammack 1996), appears to be a promising tool for tracing eukaryotic history.

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