Mitochondrial Genome of the Colorless Green Alga *Polytomella parva*: Two Linear DNA Molecules with Homologous Inverted Repeat Termini

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Most of the well-characterized mitochondrial genomes from diverse green algal lineages are circular mapping DNA molecules; however, *Chlamydomonas reinhardtii* has a linear 15.8 kb unit mitochondrial genome with 580 or 581 bp inverted repeat ends. In mitochondrial-enriched fractions prepared from *Polytomella parva* (=*P. agilis*), a colorless, naturally wall-less relative of *C. reinhardtii*, we have detected two linear mitochondrial DNA (mtDNA) components with sizes of 13.5 and 3.5 kb. Sequences spanning 97% and 86% of the 13.5- and 3.5-kb mtDNAs, respectively, reveal that these molecules contain long, at least 1.3 kb, homologous inverted repeat sequences at their termini. The 3.5-kb mtDNA has only one coding region (*nad6*), the functionality of which is supported by both the relative rate at which it has accumulated nonsynonymous nucleotide substitutions and its absence from the 13.5-kb mtDNA which encodes nine genes (i.e., large and small subunit rRNA [LSU and SSU rRNA] genes, one tRNA gene, and six protein-coding genes). On the basis of DNA sequence data, we propose that a variant start codon, GTG, is utilized by the *P. parva* 13.5-kb mtDNA-encoded gene, *nad5*. Using the relative rate test with *Chlamydomonas moewusii* (=*C. eugametos*) as the outgroup, we conclude that the nonsynonymous nucleotide substitution rate in the mitochondrial protein-coding genes of *P. parva* is on an average about 3.3 times that of the *C. reinhardtii* counterparts.

Introduction

Mitochondrial genomes in diverse lineages, despite their proposed, shared α-proteobacterial ancestry, show extensive variability in size and structural organization (Gray, Burger, and Lang 1999). For example, mitochondrial genomes in land plants encode about 50–70 genes, excluding unique and intron-encoded open reading frames (Lang, Gray, and Burger 1999; Kubo et al. 2000), range in size from about 180 to 2,400 kb, and have a very complex and not well understood in vivo structural organization (Bendich 1993; Backert, Nielsen, and Börner 1997; Oldenburg and Bendich 2001), despite the presence of physical maps which suggest that master circular forms can give rise to subgenomic circular forms by intramolecular recombination (Palmer and Shields 1984; Fauron et al. 1995; Unseld et al. 1997). In comparison, mitochondrial genomes in the protozoan genus *Plasmodium*, the causative agent of malaria, encode only five genes in a 6-kb element which is repeated in variably sized tandem arrays (Feagin 1994; Wilson and Williamson 1997). Moreover, examples of subgenomic, presumably autonomously replicating, circular or linear mitochondrial DNA (mtDNA) forms that encode standard mitochondrial genes have been identified in some mesozoan (Watanabe et al. 1999) and metazoan (Bridge et al. 1992; Armstrong, Blok, and Phillips 2000; Pont-Kingdon et al. 2000) animals. These mtDNAs differ from the special classes of small circular mtDNA molecules found in the single mitochondrion (kinetoplast) of trypanosomal protozoa that encode only guide RNAs used to edit transcripts produced by the main mtDNA (Shapiro and Englund 1995) and the senDNA or other subgenomic circular mtDNA forms associated with senescent or particular mutant strains of filamentous fungi, respectively (Griffiths 1992).

To date, mitochondrial genomes from eight taxa representing diverse lineages of green algae (Chlorophyta *sensu* Sluiman 1985) have been sequenced to completion (reviewed by Lang, Gray, and Burger 1999; Turmel, Otis, and Lemieux 2002). Seven of these taxa have circular mapping mtDNAs, whereas the remaining taxon *Chlamydomonas reinhardtii*, a member of the ''*Volvox* clade'' (*sensu* Nakayama et al. 1996) of the class Chlorophyceae (*sensu* Mattox and Stewart 1984), has a linear 15.8-kb mtDNA with a 580- or 581-bp sequence at one terminus that is repeated in an inverted orientation at the other terminus (Vahrenholz et al. 1993). Mitochondrial genomes from other members of the *Volvox* clade that have been characterized by gel electrophoresis are also linear mtDNAs (Moore and Coleman 1989; unpublished data). To date, there is no evidence among the green algae of a mitochondrial gene being associated with a subgenomic mtDNA such as found in some lineages outside of this group.

The genus *Polytomella* is composed of a morphologically and physiologically homogeneous group of colorless and wall-less unicells (Pringsheim 1955) which appear to have arisen from a green ancestor within the *Volvox* clade (Nakayama et al. 1996) of chlorophycean green algae. The absence of both a cell wall and thylakoid membranes in *Polytomella* has facilitated the purification of mitochondrial respiratory proteins and therefore made this an attractive taxon for mitochondrial studies (Gutiérrez-Cirlos et al. 1994; Atteia, Dreyfus, and González-Halphen 1997). Sequences have been reported for the mitochondrial genes, *cox1* and *cob*, from *Polytomella* strain SAG 198.80 (Antaramian et al. 1996, 1998).

In this study we describe two linear mtDNA components of *P. parva*, a 13.5-kb mtDNA which contains most of the standard mitochondrial-coding sequences present in *C. reinhardtii* mtDNA and a 3.5-kb mtDNA

Key words: *Polytomella parva*, green algae, subgenomic mitochondrial DNA, nonstandard start codon, evolutionary rate.

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Mol. Biol. Evol. 19(7):999–1007. 2002

which contains only one gene, *nad6*, a gene which is absent from the 13.5-kb mtDNA. Both DNA components contain long-terminal inverted repeat sequences which are almost identical between the 13.5- and 3.5-kb linear mtDNAs but show no similarity with the terminal inverted repeat sequences in the linear mtDNA of *C. reinhardtii*. This is the first report of subgenomic mtDNA in green algae.

Materials and Methods

Strain, Culture Conditions, Mitochondrial Isolation, and DNA Isolation

Polytomella parva (UTEX L 193) was obtained from the University of Texas at Austin culture collection and routinely checked to ensure the absence of microbial contaminants. Cells were cultured at 25°C in the medium of Sheeler, Cantor, and Moore (1968), with shaking for small cultures (100–250 ml) or mild aeration for larger cultures (5–15 liter) and harvested in the late logarithmic phase of growth ($OD_{750\ nm} = 0.45$) by centrifugation (2,000*g*) at 4°C. Mitochondrial-enriched fractions were prepared and treated with DNase I (code DPRF, Worthington) following procedure B of Ryan et al. (1978).

The isolation of DNA followed the method of Ryan et al. (1978) with the following exceptions. Whole cell and mitochondrial-enriched pellets were lysed in 2% sarkosyl, 1% SDS, 1 mg/ml proteinase K (Boehringer Mannheim) at 50°C for 1 h. After the RNase treatment step and the final extraction with chloroform-isoamyl alcohol, some remaining non-DNA materials were removed by precipitation at room temperature in the presence of 2.5 M ammonium acetate. DNA was then precipitated twice with ethanol and redissolved in 10 mM Tris-HCl (pH 8.0)-1 mM EDTA. There was no further DNA purification step employing preparative CsCl gradient centrifugation as described by Ryan et al. (1978).

DNA Amplification

PCR experiments were performed in a thermal cycler (Geneamp PCR System 2400, Perkin-Elmer) using total cellular DNA as the template and reagents from MBI Fermentas. DNA was initially denatured at 94°C for 3 min and amplified by 40 cycles, each involving denaturation at 94°C for 45 s, annealing at 50°C for 30 s, and extension at 72°C for 2 min; there was a final extension period at 72°C for 7 min. PCR products were purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech).

DNA Transfer and Southern Blot Hybridization

After fractionation by agarose (1%) gel electrophoresis (6 V/cm), DNA was transferred to a nylon membrane (Hybond N^+, Amersham Pharmacia Biotech) using the capillary transfer method (Sambrook, Fritsch, and Maniatis 1989, pp. 9.34–9.35) with 0.5 M NaOH as the transfer solution. The Alkphos Direct Labelling and Detection System kit (Amersham Pharmacia Biotech) was used for DNA probe labeling, and the subsequent hybridization followed the recommended protocol, except that hybridization was carried out overnight at 60°C. Chemiluminescent detection was achieved by exposing the autoradiographic film to the membrane. For reprobing, the membrane was stripped in 0.5% SDS and checked for completeness of signal removal.

MtDNA Cloning

DNA isolated from a mitochondrial-enriched preparation was fractionated by agarose (1%) gel electrophoresis. After being stained with ethidium bromide, the 13.5- and 3.5-kb bands, presumed to be mtDNAs, were cut from the gel, and the DNA was recovered using the GFX PCR DNA and Gel Band Purification kit (Amersham Pharmacia Biotech). The recovered 13.5 and 3.5 kb DNAs were digested with *Hin*dIII and *Eco*RI, respectively, and then ligated into the *Hin*dIII or *Eco*RI site of the vector pBluescript II SK^+ (Stratagene). The ligation mixture was used to transform *Escherichia coli* strain XL1-Blue MCF'(Stratagene). Recombinant plasmids were extracted from the host cells by the alkaline lysis preparation method (Sambrook, Fritsch, and Maniatis 1989, pp. 1.25–1.28), and the recombinant plasmids containing inserts of the 13.5- and 3.5-kb mtDNAs were identified by Southern blot hybridization using these DNAs as probes.

DNA Sequencing

Cloned, and in some cases PCR amplified, mtDNA segments were sequenced commercially (Dalhousie University—NRC Institute for Marine Biosciences Joint Laboratory, Halifax, or Center for Applied Genomics, Hospital for Sick Children, Toronto) on both strands using LICOR 4200 (LICOR; dye primers) or ABI 373 or 377 (PE-Applied Biosystems; dye terminators) automated DNA sequencers. The sequence of DNA amplified by PCR was obtained with the PCR product or two independent clones of the product.

Data Analysis

The BLAST network services (Altschul et al. 1990) provided at the National Center for Biotechnology Information were used for sequence similarity searches. The program Gene Runner (Hastings Software) was used for sequence editing and compiling. Multiple DNA and protein sequence alignments were performed using the program CLUSTAL W, version 1.7 (Thompson, Higgins, and Gibson 1994). The program RRTree, version 1.1.10 (Robinson-Rechavi and Huchon 2000) was employed for nucleotide substitution analyses and relative rate tests.

Results

Identification and General Features of the Two MtDNA Components

After fractionation by agarose gel electrophoresis, DNA from the mitochondrial-enriched fraction of *P. parva* revealed two prominent components that were

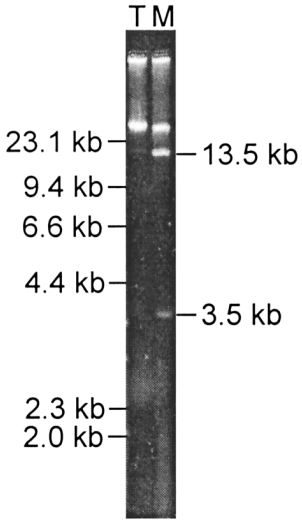

FIG. 1.—Agarose (1%) gel electrophoresis of *P. parva* DNA isolated from total cellular (T) and mitochondrial-enriched (M) fractions. The DNA sizes indicated are based on lambda DNA *Hin*dIII and *Bst*EII fragments (MBI Fermentas).

barely visible or not visible, respectively, in the total cellular DNA preparation. The two components consistently corresponded to sizes of 13.5 and 3.5 kb, relative to linear DNA size markers when the concentration of agarose was either 1% (fig. 1) or 0.6% (data not shown). These results support the linear conformation of the two DNA species (Johnson and Grossman 1977). By means of genomic DNA cloning, and in some cases PCR amplification, DNA segments collectively spanning 97% and 86% of the 13.5- and 3.5-kb DNAs, respectively, were recovered and sequenced, thereby yielding two partial physical and gene maps (fig. 2). The inverted repeat structure of the termini of the two partial maps, together with our inability to recover either clones or PCR products bridging the ends of the 13.5- or 3.5-kb molecules, further argue that these DNAs are linear molecules with unique ends. Considering the source of the two DNAs and the coding regions they contain, it is concluded that the 13.5- and 3.5-kb DNAs are components of the *P. parva* mitochondrial genome.

Coding regions identified in the two *P. parva* mtDNAs include seven respiratory chain protein–coding genes, one tRNA gene, and LSU and SSU rRNA genes (table 1). All these coding regions are in the 13.5-kb mtDNA, except for *nad6*, which is in the 3.5-kb mtDNA. As in the mtDNA of *C. reinhardtii* (reviewed by Michaelis, Vahrenholz, and Pratje 1990), the coding regions in the *P. parva* 13.5-kb mtDNA are compactly organized, intron-free, and arranged into two unequally sized clusters, one of which is in the opposite transcriptional orientation from the other.

Although most of the protein-coding genes in the two identified mtDNAs of *P. parva* appear to have a standard ATG start codon, we infer from the DNA sequence data that mitochondria of this taxon utilize an unusual start codon for *nad5*. Two initiation codons

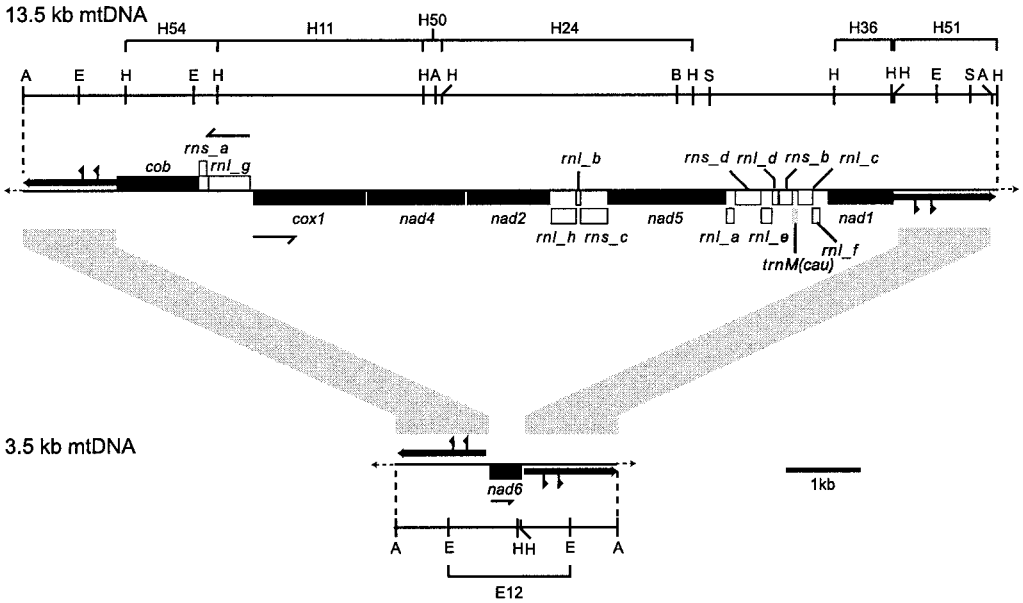

FIG. 2.—Partial physical and gene maps of *P. parva* 13.5- and 3.5-kb mtDNAs based on sequences obtained from 13,135 bp of the 13.5-kb mtDNA and 3,018 bp of the 3.5-kb mtDNA. Most of these sequences were obtained from cloned *Hin*dIII or *Eco*RI restriction fragments of the two mtDNAs, indicated above and below the respective maps. The specific linkage of H54, H11, H50, and H24 in the 13.5-kb mtDNA is based on gene continuity between the fragments. The sequences of the two internal uncloned regions of the 13.5-kb mtDNA were obtained from PCR products that were produced with primers designed from the flanking cloned fragments. The sequences flanking the left terminus of the 13.5-kb mtDNA and the two termini of the 3.5-kb mtDNA were obtained from PCR products produced with primer pairs, including in each case an outside primer designed from the outermost region of fragment H51 and an inner sequence of the closest cloned region. For gene abbreviations see table 1. Half arrows indicate directions of gene transcription. Thick solid arrows near the ends of the maps denote terminal inverted repeats; the two flags within these regions represent two direct subrepeats. Shading depicts the homologous feature of the repeat sequences between the two mtDNAs. Dashed arrows at the very ends of the maps represent the predicted and unsequenced termini of the 13.5- and 3.5-kb mtDNAs. Restriction sites shown are: A, *Ava*I; B, *Bgl*I; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I.

Table 1
Coding Regions[a] Identified in the Mitochondrial Genome of *P. parva*

A.	Ribosomal RNA genes[b] (2)
	Small subunit rRNA in pieces *rns_a, rns_b, rns_c, rns_d*
	Large subunit rRNA in pieces *rnl_a, rnl_b, rnl_c, rnl_d, rnl_e, rnl_f, rnl_g, rnl_h*
B.	Transfer RNA genes (1)
	trnM(cau)
C.	Respiratory chain genes (7)
	NADH dehydrogenase (*nad1, nad2, nad4, nad5, nad6*)
	apocytochrome *b* (*cob*)
	cytochrome oxidase (*cox1*)

[a] All the coding regions are located in the 13.5-kb mtDNA, except for *nad6*, which is encoded in the 3.5-kb mtDNA.
[b] The rDNA modules are named in the 5′–3′ order in which their transcript counterparts appear in conventional LSU and SSU rRNAs.

have been proposed for *C. reinhardtii nad5*, with the one of Boer and Gray (1986) being 63 nucleotides downstream of the one proposed by Vahrenholz et al. (1985). Pairwise alignment of the derived amino acid sequences of *P. parva* and *C. reinhardtii nad5* (data not shown) is consistent only with the downstream start codon position of this gene in *C. reinhardtii*; however, the *P. parva* gene revealed a GTG rather than an ATG codon at the corresponding position.

Polytomella parva 13.5-kb mtDNA shares the feature of discontinuous and scrambled LSU and SSU rRNA coding regions with all other well characterized chlorophycean green algal mtDNAs (Nedelcu et al. 1996, 2000 and references therein). In the case of *P. parva*, the LSU and SSU rRNA genes are disrupted into at least eight (*rnl_a* through *_h*) and four (*rns_a* through *_d*) modules, respectively (table 1 and fig. 2). Interestingly, *rns_a* and *rnl_g* are located transcriptionally opposite to the rest of the LSU and SSU rDNA modules and thus show a feature not previously reported in the mtDNA of green algae; however, rRNA-coding regions in the apicomplexans *Plasmodium* and *Theileria* are distributed on both DNA strands (Feagin 1994).

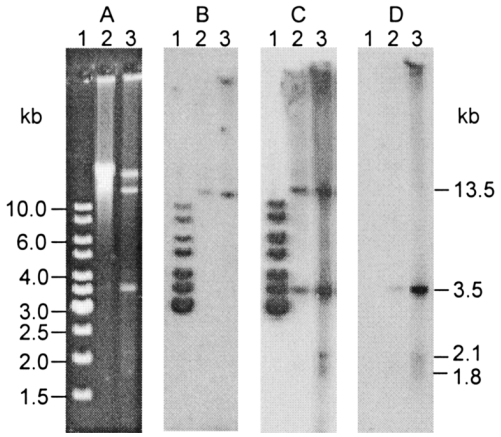

FIG. 3.—Southern blot hybridization analysis of the *P. parva* 13.5- and 3.5-kb mtDNAs. *A*, ethidium bromide staining pattern and *B*, *C*, and *D*, Southern blot analysis with clone H54, clone E12, and a PCR product of *nad6*, respectively. DNA markers derived from plasmid digests (MBI Fermentas) (lane 1), total cellular DNA (lane 2), and DNA extracted from a mitochondrial-enriched pellet (lane 3) were fractionated by agarose (1%) gel electrophoresis. Homology between the vector sequence of clones H54 and E12 and the plasmid-derived DNA markers accounts for the hybridization signals associated with these markers in *B* and *C*.

Southern blot hybridization experiments of *P. parva* total cellular and mitochondrial-enriched DNA preparations with *P. parva* mtDNA probes confirm the homology between the termini of the 13.5- and 3.5-kb mtDNA maps as well as the absence of *nad6* from the 13.5-kb mtDNA. The clone containing the 13.5-kb mtDNA fragment H54, which contains *cob*, *rns_a*, and part of *rnl_g*, detected the 13.5- but not the 3.5-kb mtDNA. The clone containing the 3.5-kb mtDNA fragment E12 (fig. 2), which contains part of the two inverted repeat regions and *nad6*, detected both the 13.5- and the 3.5-kb mtDNA components. Finally, the PCR product, derived from *nad6*, detected the 3.5- but not the 13.5-kb mtDNA (fig. 3). The last two probes gave additional discrete signals in the mitochondrial-enriched DNA preparation (and in more exposed blots of total cellular DNA) at positions corresponding to linear DNA molecules of about 2.1 and 1.8 kb. These results, which have been observed consistently with independent DNA samples, imply the existence of additional small mtDNA molecules that harbor *nad6* sequence.

Flanking Sequences of the 13.5- and 3.5-kb MtDNAs

The alignment of the available terminal sequences derived from the 13.5- and 3.5-kb mtDNAs reveals a homologous inverted repeat sequence of almost 1.3 kb in the two DNAs (fig. 4). The left and right repeat sequences in the 13.5-kb mtDNA and the right repeat sequence in the 3.5-kb mtDNA start immediately downstream of *cob*, *nad1*, and *nad6*, respectively. The left repeat of the 3.5-kb mtDNA starts 43 bp upstream of *nad6*, and a stem-loop structure (not shown) can be modeled from this 43 bp sequence. The four copies of the repeat sequence show only occasional differences in sequence, and a 44-bp sequence present in the right repeat of the 13.5-kb mtDNA is missing from the other three copies of the repeat. It is noteworthy that single copies of the 7-bp sequence 5′-TGCGCAC-3′ are located at one end of and immediately following the other end of this extra 44 bp sequence, thereby suggesting its loss from the remaining three terminal repeat regions by unequal

FIG. 4.—Multiple alignment of the available flanking sequences in the 13.5- and 3.5-kb mtDNAs of *P. parva*. Sequences begin immediately (*a*) downstream of *cob* in the 13.5-kb mtDNA, (*b*) downstream of *nad1* in the 13.5-kb mtDNA, (*c*) upstream of *nad6* in the 3.5-kb mtDNA, and (*d*) downstream of *nad6* in the 3.5-kb mtDNA. A 43-bp sequence immediately upstream of *nad6*, that is missing from the other three repeat regions, can be modeled into a stem-loop structure. A 44-bp sequence is present in the repeat region downstream of *nad1*, that is missing from the other three repeat regions; single copies of a 7-bp sequence located at one end of and immediately following the other end of this extra 44 bp sequence are underlined. Shaded areas indicate two direct repeats within each copy of the inverted repeat sequence. Asterisks indicate positions in which an identical nucleotide appears in all the four sequences.

crossing over, intrastrand deletion, or slipped-strand mispairing (Graur and Li 2000, pp. 32–35). Interestingly, all four copies of the terminal repeat sequence contain two copies of a 42-bp direct subrepeat which are separated from each other by 197 bp. No open reading frame having a potential coding capacity of more than 70 amino acids was detected in the sequenced part of the inverted repeat regions. After

Table 2
Differences in the Number of Nonsynonymous Substitutions per 100 Sites and the Relative Rates of Nonsynonymous Substitutions in Mitochondrial Genes Between *P. parva* (species 1) and *C. reinhardtii* (species 2) with *C. moewusii* (species 3) as a Reference

Genes	Nucleotides Compared	K_{12}[a]	K_{13}[a]	K_{23}[a]	$K_{13} - K_{23} \pm SE$[b]	Rate Ratio[c]
cob	1110	38.2	37.0	20.3	16.6 ± 2.7	2.5
cox1 . . .	1503	20.7	26.1	16.4	9.7 ± 1.7	2.8
nad1 . . .	873	41.1	42.5	16.5	26.0 ± 3.3	4.4
nad2 . . .	1095	85.4	88.0	39.4	48.5 ± 7.0	3.6
nad4 . . .	1302	62.8	67.7	31.4	36.3 ± 4.1	3.7
nad5 . . .	1569	59.3	62.7	33.2	29.5 ± 3.5	3.0
nad6 . . .	432	65.9	62.7	31.8	30.9 ± 6.8	2.8

[a] K_{ij} = number of nonsynonymous substitutions per 100 nonsynonymous sites between species i and j.

[b] SE = standard error.

[c] The ratio of the rate in the *P. parva* lineage to the rate in the *C. reinhardtii* lineage (D. Graur and W.-H. Li 2000, pp. 142–143).

BLAST searches, no sequence in any of these regions was found to be significantly similar to any sequence in the GenBank at the level of either protein or DNA.

Evolutionary Rate Analysis

Nucleotide substitution levels for seven protein-coding genes encoded in mtDNA were estimated for all pairwise comparisons between homologs of *P. parva*, *C. reinhardtii*, and *Chlamydomonas moewusii* (=*C. eugametos* UTEX 9). Levels of synonymous substitution were saturated between all homologous gene sequences and therefore could not be calculated. Differences in the number of nonsynonymous substitutions were estimated (table 2), and these were used to calculate the rate of nonsynonymous substitution between homologous mitochondrial genes in the *P. parva* lineage relative to the *C. reinhardtii* lineage using *C. moewusii* as the outgroup (Buchheim et al. 1996; Nakayama et al. 1996). The value of K_{13} (number of nonsynonymous substitutions between *P. parva* and *C. moewusii*) − K_{23} (number of nonsynonymous substitutions between *C. reinhardtii* and *C. moewusii*), for each of the mitochondrial genes compared, is consistently positive and more than five times the standard error, indicating that the nonsynonymous substitution rate difference between the *P. parva* and *C. reinhardtii* lineages for these genes is highly significant. The nonsynonymous substitution rate of the protein-coding genes in the *P. parva* lineage averages about 3.3 times that of the homologs in the *C. reinhardtii* lineage, with the lowest value being 2.5 times for *cob* and the highest value being 4.4 times for *nad1*. Interestingly, *nad6*, the only gene identified in the 3.5-kb mtDNA, has a nonsynonymous substitution rate ratio of 2.8 between the two lineages which is not remarkable compared with the other mitochondrial genes characterized.

Discussion

Genome Structure

We have identified and partially characterized two linear mtDNA components from *P. parva* which have sizes of 13.5 and 3.5 kb. This represents the first description of subgenomic mtDNAs from a green alga. The standard, apparently required gene, *nad6*, is the only gene identified in the 3.5-kb mtDNA; the absence of *nad6* from the 13.5-kb mtDNA argues for the function of this DNA. Moreover, for *nad6*, the ratio of the number of nonsynonymous substitutions in the *P. parva* lineage to that in the *C. reinhardtii* lineage is within the range of the other mtDNA-encoded genes, thus supporting the view that *nad6* is under normal evolutionary constraints and therefore functional. A potential stem-loop structure in the region immediately upstream of *nad6* might play some role in the initiation of *nad6* transcription because similar potential structures have been identified upstream of genes in the minicircular mtDNAs of the mesozoan animal *Dicyema misakiense* (Watanabe et al. 1999). Interestingly, no similar potential structure was identified in the 13.5-kb mtDNA molecule of *P. parva*. We did, however, identify two potential promoter sequences for bidirectional transcription initiation between *rnl_g* and *cox1* in *P. parva* 13.5-kb mtDNA. One, 5′-ATATTCTTA-3′, is located nine nucleotides upstream of *cox1* and the other, 5′-GTATTGCTG-3′, is located five nucleotides upstream of *rnl_g*. These sequences show similarity with the consensus promoter sequence in the mtDNA of fungi (Tracy and Stern 1995) as well as the potential promoters identified upstream of *cox1* and *nad5* (Duby et al. 2001) in the region of bidirectional transcription initiation proposed for *C. reinhardtii* mtDNA (Gray and Boer 1988).

Examples of subgenomic mtDNAs, most of which are circular mapping, have been described in other eukaryotic lineages, with varying degrees of completeness. *Polytomella parva*, however, seems to offer the only clear example of a mitochondrial genome containing subgenomic linear DNA molecules that harbor standard mitochondrial-coding regions and that share homologous inverted repeat ends. Certain hydrozoan taxa have been shown to contain two ca. 8-kb linear mtDNA molecules, in contrast to the single 14- to 17-kb linear mtDNA found in most hydrozoans (Warrior and Gall 1985; Bridge et al. 1992); nevertheless, except for a 3.2-kb sequence at an end of one of the two linear mtDNAs from *Hydra attenuata* (Pont-Kingdon et al. 2000), these genomes are not well characterized.

The gene content and discontinuous structure of the rRNA-coding regions identified in the *P. parva* mitochondrial genome is typical of the reduced-derived type of mtDNA (Gray, Burger, and Lang 1999) identified in *C. reinhardtii* (Boer and Gray 1988*a*, Michaelis, Vahrenholz, and Pratje 1990), *Chlorogonium capillatum* (= *C. elongatum* SAG 12-2e) (Kroymann and Zetsche 1998), and *C. moewusii* (UTEX 9) (Denovan-Wright, Nedelcu, and Lee 1998), except for two tRNA genes, *trnW*(*cca*) and *trnQ*(*uug*), not identified in the *P. parva* mtDNA; a reverse transcriptase–like coding region (*rtl*)

(Boer and Gray 1988*b*), possibly a degenerate group-II intron (Nedelcu and Lee 1998), so far identified only in the mtDNA of *C. reinhardtii* has also not yet been detected in the *P. parva* mtDNA. The missing tRNA-coding regions could not be identified in the sequenced portion of the two *P. parva* mtDNAs using the program tRNAscan SE 1.21 (Lowe and Eddy 1997), and there appears to be no remaining space outside of the inverted repeat sequence regions of the 13.5- and 3.5-kb mtDNAs that could accommodate the expected ca. 75 bp coding regions. Moreover, we suggest that the short DNA segments currently unsequenced at each end of the two identified *P. parva* mtDNAs are also part of the terminal inverted repeats and have no coding function. Although transfer of *trnW*(*caa*) and *trnQ*(*uug*) to the nucleus in *P. parva* is possible, an alternative explanation is that they are encoded in one or two additional as yet unidentified mtDNA(s).

On the basis of the available evidence, we cannot decide conclusively at the present time whether or not the 13.5- and 3.5-kb mtDNAs of *P. parva* replicate autonomously. In our Southern blot hybridization experiments (fig. 3), probes specific to the 13.5- or 3.5-kb mtDNAs both revealed signals in the well regions of the gel almost equivalent in intensity to those of the migrating 13.5 and 3.5 kb components. These signals could have resulted from (1) 13.5- and 3.5-kb linear mtDNA molecules that were trapped in the well regions possibly by nuclear DNA or impurities (or both), or (2) one or more larger replicative forms of mtDNA from which the 13.5- and 3.5-kb sequences are normally excised. We favor the former possibility because of our inability to obtain PCR products connecting the 13.5- and 3.5-kb mtDNAs or bridging the ends of each of these DNA components.

If the 13.5- and 3.5-kb linear DNAs are not derived from some larger replicative form(s) and they replicate autonomously as linear molecules, they would require a mechanism to replicate their 5′-ends like any other linear DNA capable of replication. mtDNA telomeres from a variety of organisms have evolved a diversity of mechanisms aimed at solving this problem as revealed by their distinct structures (reviewed by Nosek et al. 1998). The available information does not enable us to propose a specific telomeric mechanism that might be employed by the mtDNAs of *P. parva*, and it is unclear as to the possible role in this potential process, if any, that could be played by the direct subrepeat sequences common to the four copies of the inverted repeat sequence. In the absence of sequence at the very termini of the 13.5- and 3.5-kb mtDNAs, we cannot rule out the possibility that these direct subrepeat sequences share sequence identity with the outermost termini of the *P. parva* mtDNAs and have a role in telomere maintenance, as proposed for the internal 86 bp repeat of the outermost inverted repeat sequence in *C. reinhardtii* mtDNA (Vahrenholz et al. 1993; Duby et al. 2001).

Elevated Evolutionary Rate

On an average, the nonsynonymous substitution rate in the mitochondrial protein-coding genes is about 3.3 times greater in the *P. parva* lineage compared with the *C. reinhardtii* lineage. *Polytomella parva* mitochondrial protein-coding genes, therefore, in terms of nucleotide substitutions that cause amino acid change, seem to be evolving conspicuously faster than those in *C. reinhardtii*. Interestingly, the same trend is observed in phylogenetic trees based on 18S rDNA sequences (Nakayama et al. 1996); therefore, this suggests that the higher evolutionary rate is characteristic of the *P. parva* lineage rather than a particular genetic compartment of the lineage. Such a lineage effect could be explained by (1) a greater number of mutations, potentially because of a greater number of generations, or (2) a higher probability of mutation fixation, possibly because of a smaller population size (Pringsheim 1955), or both, relative to the *C. reinhardtii* lineage. It is noteworthy that an accelerated rate of evolution in rRNA genes residing in the nuclear, mitochondrial, and plastid compartments has also been observed in some nonphotosynthetic holoparasitic plants (Wolfe et al. 1992; Duff and Nickrent 1997 and references therein).

Nonstandard Start Codon

On the basis of the DNA sequence, a nonstandard start codon, GTG, is predicted in *nad5* of *P. parva* 13.5-kb mtDNA. Evidence has been reported that mitochondria in several lineages use nonstandard initiation codons, and in many cases this includes GTG, as, for example, in the protist *Tetrahymena pyriformis* (Edqvist, Burger, and Gray 2000). Although there is no previous report for the use of unusual start codons in green algal mitochondria, other nonstandard codons appear to be used (Hayashi-Ishimaru et al. 1996; Turmel et al. 1999; Kück, Jekosch, and Holzamer 2000; Nedelcu et al. 2000). The possibility that G to A editing could modify the GTG codon to ATG in the *nad5* transcript of *P. parva* has not been formally eliminated; however, this seems unlikely at the present time because RNA editing has not yet been reported in green algal mitochondria, and G to A editing is rare, having only recently been detected in HIV-1 viral transcripts (Bourara, Litvak, and Araya 2000).

Supplementary Material

The partial sequences of the *P. parva* 13.5- and 3.5-kb mtDNAs are registered under GenBank accession numbers AY062933 and AY062934, respectively.

Acknowledgments

We thank Murray Schnare for suggestions, assistance with experiments, encouragement throughout the course of this work, and for his detailed editing of an earlier version of this paper. We also acknowledge the helpful discussions with Gertraud Burger, Michael Gray, Mark Laflamme, and Michael Reith, and the preparation of figures by Mark Laflamme. This work was supported by a research grant to R.W.L. from the Natural Sciences and Engineering Research Council of Canada. Partial financial support was provided to J.F. by Dalhousie University and Patrick Lett Scholarships.

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KEN WOLFE, reviewing editor

Accepted January 24, 2002