New Phytologist

Letters

Does mitochondrial DNA replication in *Chlamydomonas* require a reverse transcriptase?

Introduction

Among the first green algal mitochondrial DNAs (mtDNAs) to be described in great detail was that of *Chlamydomonas reinhardtii* (Grant & Chiang, 1980; Gray & Boer, 1988; Vahrenholz *et al.*, 1993). It is a *c.* 16 kb linear molecule containing fragmented rRNA genes and telomeres, arranged in an inverted-repeat orientation with 3′ single-stranded overhangs (Fig. 1a). Another intriguing feature of this genome is a free-standing open reading frame (1119 nt) encoding a reverse transcriptase-like protein (the *rtl* gene), which is believed to have originated from an ancient group II intron (Boer & Gray, 1988) but is no longer associated with one. Comparative genomics and transcriptomics have shown that *rtl* is under purifying selection (albeit weaker than that of the standard mitochondrial protein-coding genes), is transcriptionally active and undergoes posttranscriptional processing, suggesting that this gene is functional (Boer & Gray, 1988; Popescu & Lee, 2007; Smith & Lee, 2008a; Salinas-Giegé *et al.*, 2017; Gallaher *et al.*, 2018). Its exact role remains unknown.

Vahrenholz *et al.* (1993) proposed that *rtl* is involved in *C. reinhardtii* mtDNA replication, which if true would be unprecedented for an organelle genome. Their hypothesis was based on the fact that an 86-nt stretch of internal mtDNA matches perfectly to the extreme termini of the linear genome, including the 3′ overhangs (Fig. 1a), that is the outermost 86 nt of the two telomeres is repeated once within the mtDNA. This short internal telomeric repeat (or part of it), they suggested, could be transcribed and the resulting RNA used to prime DNA synthesis at the ends of the genome, giving rise to two nearly complete mtDNA molecules (Fig. 1b; Supporting Information Fig. S1). A reverse transcriptase enzyme, generated by *rtl*, could then complete the sequences of the ends by copying the RNA primers of the parental strands (Figs 1b, S1). The beauty of this model is that it not only provides a straightforward solution to how the *C. reinhardtii* mtDNA might replicate but also simultaneously explains the presence of *rtl*, the 3′ telomeric extensions and the internal telomeric repeat.

Although nearly three decades have passed since Vahrenholz and colleagues published this model, few new insights have been gained into the mode of mtDNA replication in *C. reinhardtii* and the role, if any, of *rtl*. However, two interesting findings are notable. First, mitochondrial transformation experiments have shown that the presence of the entire left telomere, including the 3′ overhang, is essential to reach a high level of transformation (Duby *et al.*, 2001; Remacle *et al.*, 2006; Larosa, 2012). Unfortunately, the current *C. reinhardtii* mitochondrial transformation system does not allow for modification of the right telomere or *rtl*. Second, detailed transcriptional profiling of the *C. reinhardtii* mtDNA has revealed that an RNA transcript is generated from the internal telomeric repeat, specifically from the antisense strand (Salinas-Giegé *et al.*, 2017), meaning the transcript has the correct polarity to bind to the 3′ overhangs and act as a primer for DNA synthesis on both parental strands (Figs 1b, S1).

Recent years have seen the sequencing of mtDNAs from dozens of other chlamydomonadalean species. Most of these genomes are circular mapping and lack *rtl* (Smith *et al.*, 2013; Hu *et al.*, 2019; Zhang *et al.*, 2019), but some are linear. The colonial alga *Yamagishiella unicocca*, for instance, has a linear mitogenome with inverted-repeat telomeres, short internal telomeric repeats and *rtl*, but the structure of the chromosome ends (e.g. 3′ overhangs) is undetermined (Hamaji *et al.*, 2017). *Eudorina* sp. NIES-3984 is a close relative of *Y. unicocca* and both algae have identical mitochondrial gene orders. *Eudorina* sp., however, has a primarily circularly mapping genome and, most importantly, is missing *rtl* (Hamaji *et al.*, 2017), supporting the notion that this gene is involved in mitochondrial telomere maintenance. The only other well-studied example of linear mtDNAs from the Chlamydomonadales is *Polytomella*. All known members of this nonphotosynthetic genus harbour linear mitogenomes, but they do not contain *rtl* or any internal copies of the telomeric repeats (Smith *et al.*, 2010). *Polytomella* mitogenomes terminate in single-stranded hairpins (Smith & Lee, 2008b), meaning that mtDNA replication could easily proceed via a single-stranded circular intermediate and, therefore, is not necessarily dependent on a reverse transcriptase unlike the *rtl*-based replication model for *C. reinhardtii* by Vahrenholz and colleagues.

The idea that *rtl* is involved in mtDNA replication is still a compelling one. From a comparative genomics perspective, it would be useful to have mtDNA sequences from very close relatives of *C. reinhardtii* to see if certain features are conserved such as an internal telomeric repeat. Surprisingly, these kinds of data are lacking. Only a partial mitogenome sequence is available for *Chlamydomonas incerta* (Popescu & Lee, 2007), the nearest known noninterfertile relative of *C. reinhardtii* (Schlösser *et al.*, 1976; Pröschold *et al.*, 2001), and no mtDNA sequences are available for either *Chlamydomonas schloesseri*, the closest identified sister lineage to the *C. reinhardtii*–*C. incerta* clade (Pröschold *et al.*, 2018; Nakada *et al.*, 2019) or from strains of *Chlamydomonas debaryana* (also known as *Edaphochlamys debaryana*) that consistently group close to *C. reinhardtii* in phylogenetic analyses (Yumoto *et al.*, 2013; Pröschold *et al.*, 2018; Nakada *et al.*, 2019; Craig *et al.*, 2020). In fact, the nearest relatives of *C. reinhardtii* to have their

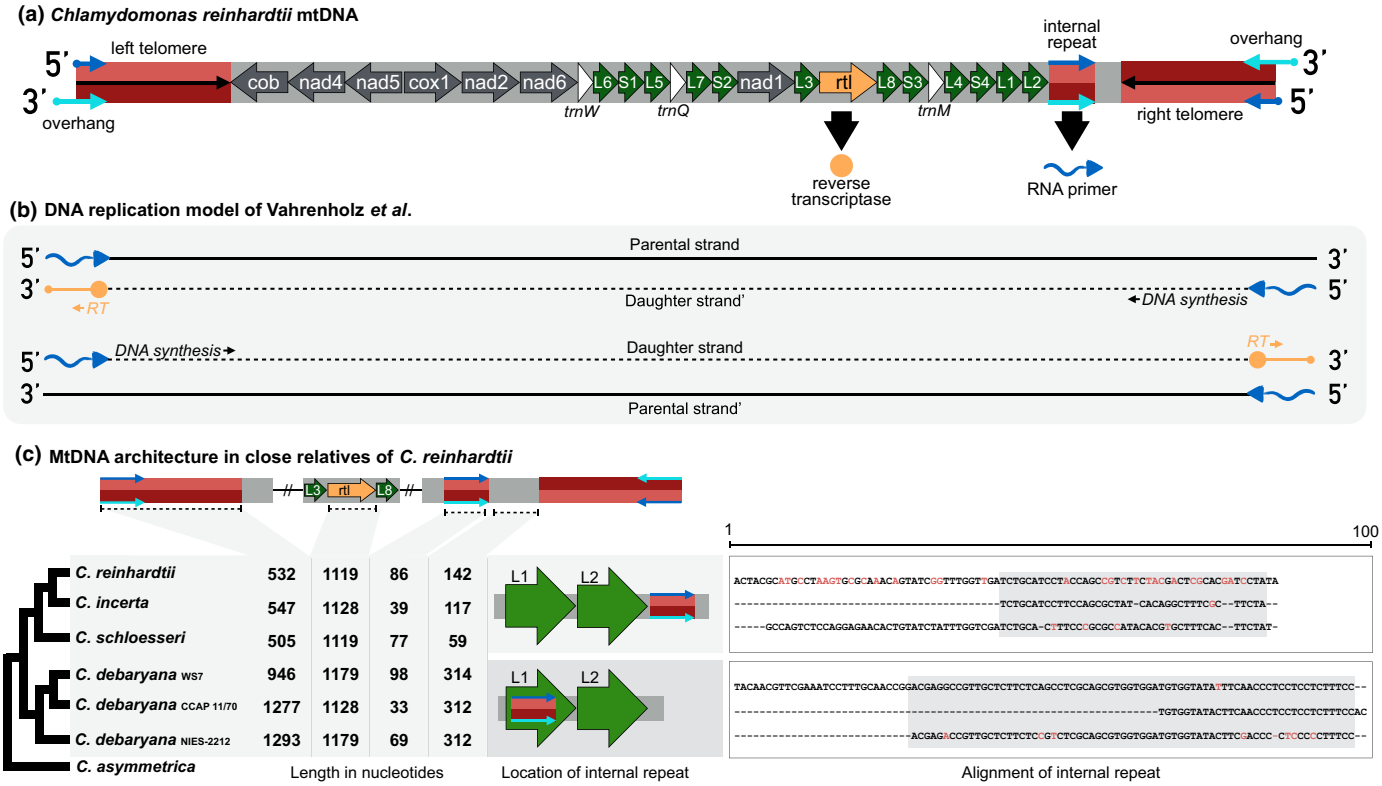

Fig. 1 Mitochondrial telomeres and their hypothetical mode of replication in *Chlamydomonas* species. (a) Mitochondrial genome map of *Chlamydomonas reinhardtii* (not to scale); the large and small subunit rRNA genes are fragmented and scrambled throughout the genome (L1–L8 and S1–S4, respectively). As in a previous study (Smith & Lee, 2008b), the so-called rRNA-coding modules L2b and L3a were considered to be intergenic regions. (b) *Chlamydomonas reinhardtii rtl*-based mitochondrial genome replication model of Vahrenholz *et al*. (1993). Blue squiggle arrows are RNA primers; yellow lines show reverse transcription reactions. Please see Supporting Information Fig. S1 for a detailed breakdown of this model. (c) mtDNA telomeres and internal repeat organization in close relatives of *C. reinhardtii*. Branching order of the tree based on previous phylogenetic analyses (Yumoto *et al*., 2013; Pröschold *et al*., 2018; Nakada *et al*., 2019; Nelson *et al*., 2019; Craig *et al*., 2020). Simplified mtDNA map (shown above the tree) is not to scale. *Chlamydomonas asymmetrica* has circular mtDNA without *rtl*. All other species have identical gene orders and contents as in the map shown in (a). Alignments of internal repeat element: differences between species/strains are shown in red and conserved regions are shaded grey.

mitogenomes sequenced are colonial algae (e.g. *Tetrabaena socialis* and *Gonium pectorale*).

Mitochondrial genomes from the closest known relatives of *C. reinhardtii*

By mining data from various ongoing nuclear genome projects and publicly available sequencing repositories (Hirashima *et al*., 2016; Nelson *et al*., 2019; Craig *et al*., 2020) (Methods S1), we collected complete or near-complete mtDNA sequences from five different *Chlamydomonas* algae (Fig. 1c): *C. incerta* SAG 7.73, *C. schloesseri* CCAP 11/173 and *C. debaryana* strains NIES-2212, CCAP 11/70 and WS7. The mtDNAs from all of these isolates assemble as linear molecules (17.6–23.1 kb) with long inverted-repeat telomeres (505–1293 nt). Their gene contents and arrangements mirror that of *C. reinhardtii*, including the presence and position of *rtl* (Fig. 1a). The *rtl* genes varied in size from 1119–1179 nt, contained no internal stop codons or frameshift mutations and were not associated with any intronic sequences. A multiple alignment of the deduced amino acid sequences of *rtl*, including that of *C. reinhardtii*, gave an average pairwise-positive identity of 67% (BLSM2 scoring matrix), which went up significantly within the region containing the core reverse transcriptase domain (Fig. S2). In short, *rtl* appears to be a functional gene in not only *C. reinhardtii* but in its closest known relatives with linear mtDNAs. But is it involved in telomere maintenance?

The most striking finding from these newly sequenced linear mtDNAs is that, like *C. reinhardtii*, the extreme ends of the telomeres are repeated once within the genome (Fig. 1c). Equally as striking is that this internal repeat occurs at approximately the same location and arrangement in all explored strains: within the final 400 nt before the start of the right telomere and with an orientation matching that of the left telomere (and the reverse complement of the right telomere) (Fig. 1c). The length of this internal telomeric repeat ranged from 33 nt to 98 nt. In *C. reinhardtii*, *C. incerta* and *C. schloesseri* it occurred downstream of the *rnl2* coding module, whereas in the three *C. debaryana* strains it was within the *rnl1* coding module, which is adjacent to *rnl2* (Fig. 1c). Consequently, the sequences of these internal repeats can only be aligned among subsets of these six isolates (Fig. 1c). Also, it is noteworthy that the telomeric repeats themselves are highly divergent among the different strains, even between the most closely related ones. The

C. reinhardtii and *C. incerta* mitochondrial telomeres, for example, share only *c.* 65% nucleotide identity, not including gaps. This is significant because it means that it is not a specific telomeric sequence that is being maintained in these linear mtDNAs – a common feature of linear organelle genomes (Smith & Keeling, 2013) – but rather a specific genomic architecture, one in which the ends of the genome match to an internal site and one that likely existed in the most recent common ancestor of the core Reinhardtinia clade (Hamaji *et al.*, 2017).

Recurring themes in support of Vahrenholz and colleagues

Although we are confident that near-complete telomeric sequences were assembled from the *Chlamydomonas* mtDNAs presented here (Methods S1), detailed laboratory work is needed to determine the precise structure of the ends and if they are represented by 3′ overhangs. Nevertheless, a recurring theme arose from these data: linear mtDNAs within *C. reinhardtii* and its close relatives have a free-standing *rtl* gene (located between the L3 and L8 rRNA-coding modules) and an internal repeat identical to the extreme termini. This internal repeat is consistently located adjacent to the right telomere with an orientation by which its putative transcriptional product can bind to the 3′ ends of the mtDNA, initiating DNA synthesis. All of this evidence supports the *rtl*-based mtDNA replication model of Vahrenholz and colleagues.

There are two other competing models for mtDNA replication in *C. reinhardtii*, both based on the presence of the internal telomeric repeat but not requiring the existence of a reverse transcriptase. One involves the formation of a T-loop through the interaction of the left telomere and internal repeat (Larosa, 2012) (Fig. S3). Indeed, rare circular forms of the *C. reinhardtii* mtDNA, potentially generated by T-loop formation, have been identified (Ryan *et al.*, 1978; Ma *et al.*, 1992; Duby *et al.*, 2001). However, the location of the internal repeat in the three *C. debaryana* strains did not support this mechanism, as it would presumably exclude replication of the *rnl2* coding module (Fig. S3). Similar to Larosa (2012), Vahrenholz *et al.* (1993) presented an alternative DNA-based replication model to their *rtl*-dependent model in which folding of mtDNA provided an interaction between the terminal and internal telomeric repeats, which then primed DNA synthesis. A site-specific endonuclease was required in both of these models, but a free-standing gene encoding such an enzyme was not found in the mtDNA of *C. reinhardtii* or its close relatives, nor has a nuclear-encoded, mitochondrial-targeted endonuclease been identified. That said, site-specific endonucleases are encoded in the mtDNA group I introns of *C. incerta*, *C. schloesseri* and the three *C. debaryana* strains as well as in certain natural isolates of *C. reinhardtii* (Smith & Lee, 2008a).

There currently exists an exception to the rule that *rtl* only occurs in linear mtDNAs. The four-celled colonial chlamydomonadalean *Tetrabaena socialis* has a circular-mapping mitochondrial genome with a free-standing *rtl* gene, situated downstream of the *rnl3* coding module (the same location as in the linear mtDNAs) (Featherston *et al.*, 2016). The length and deduced amino acid sequence of *rtl* from *T. socialis* were similar to that of its counterparts with linear mitogenomes (Fig. S2). We re-assembled *de novo* the *T. socialis* mtDNA using the raw sequencing data of Featherston *et al.* (2016) and our results were consistent with that of a circular map. Given that the transition from a linear to a circular mitogenome architecture is likely to have occurred multiple times independently within the Chlamydomonadales (Hamaji *et al.*, 2017), it is possible that *T. socialis* recently descended from an ancestor with a linear mitogenome and an *rtl*-dependent mtDNA replication system. Mapping of *T. socialis* RNA-sequencing data (GenBank accession no. SRX3367144) to its mtDNA showed very low and limited coverage for this gene, unlike for other mitochondrial protein-coding genes; this could indicate that it is no longer essential and might soon be lost.

Therefore, the mystery of *rtl* and its function in green algal mtDNAs continues. A clear path to potentially solving this riddle would be mtDNA editing of *rtl* and the internal telomeric repeat. *C. reinhardtii* is one of the few species for which an established mitochondrial transformation system exists (Remacle *et al.*, 2006), but it is still not possibly to directly target *rtl* or the internal repeat (Larosa & Remacle, 2013). When such experiments are possible, which might be soon, the findings could be paradigm shifting.

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Author contributions

DRS wrote the initial draft of the manuscript. RJC helped assemble the mitochondrial genomes. All authors contributed to writing and revising the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 A hypothetical model for reverse-transcriptase-dependent mtDNA replication in *C. reinhardtii* and its close relatives, based on Vahrenholz *et al.* (1993).

Fig. S2 Multiple alignment of the deduced amino acid sequence of *rtl* from chlamydomonadalean algae.

Fig. S3 Mitochondrial DNA replication via T-loop formation, based on Larosa (2012).

Methods S1 Genomic data used to mine or assemble mtDNAs from *Chlamydomonas* species.

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