The chlorarachniophyte: a cell with two different nuclei and two different telomeres

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Abstract. Chlorarachniophyte algae contain a complex chloroplast derived from the endosymbiosis of a eukaryotic alga. The reduced nucleus of the endosymbiont, the nucleomorph, is located between the inner and outer pair of membranes surrounding the chloroplast. The nucleomorph of chlorarachniophytes has previously been demonstrated to contain at least three small linear chromosomes. Here we describe cloning the end of the smallest nucleomorph chromosome which is shown to carry a telomere consisting of a tandemly repeated 7 bp sequence, TCTAGGG. Using the telomere repeat as a probe, we show that nucleomorph telomeres display typical heterodisperse size distribution. The nucleomorph is shown to contain only three chromosomes with a haploid genome size of just 380 kb. All six nucleomorph chromosome termini are identical with an rRNA cisron closely linked to the telomere. The nucleomorph chromosomes thus have relatively large inverted repeats at their ends. Chromosomes from the host nucleus are shown to have a different telomere repeat motif to that of the nucleomorph chromosomes.

Introduction

Chlorarachniophytes are a group of unicellular amoeboid flagellate algae with unusual chloroplasts bounded by four membranes. These so-called chloroplasts are actually drastically reduced endosymbionts, complete with a vestigial nucleus sandwiched between the second and third chloroplast membranes (Fig. 1). This vestigial nucleus, known as the nucleomorph, contains at least three small chromosomes encoding small subunit ribosomal RNA genes (srRNA) (McFadden et al. 1994a). These nucleomorph-specific srRNA genes are phylogenetically distinct from the srRNA genes present in the main nucleus of the host cell, and their gene products are apparently incorporated into ribosomes located in the periplastidal space (a remnant of the cytoplasm of the endosymbiont situated between the second and third membranes surrounding the chloroplast) (see Fig. 1). The endosymbiont, while much reduced, still retains several hallmarks of a typical eukaryotic cell (viz. 18S rRNA genes, a nuclear membrane, and linear chromosomes).

As the nucleomorph apparently contained multiple linear chromosomes (McFadden et al. 1994a), it seemed possible these chromosomes might carry telomeres similar to other eukaryotes. Telomeres consist of simple, guanine-rich DNA sequences organised into tandem arrays at the termini of eukaryotic chromosomes. Telomeric sequences, and their associated proteins, impart stability to chromosomes protecting their ends from degradation (see Shippen 1993; Biessmann and Mason 1994 for recent reviews). Moreover, the post-replicative addition of telomere sequences is also essential in preventing shortening of linear chromosomes through successive rounds of replication. In this paper we describe the termini of the nucleomorph chromosomes, and discuss their possible function in this unusual genome.

Materials and methods

Organisms. Chlorarachniophyte strain CCMP 621 was obtained from the Culture Collection of Marine Phytoplankton, Bigelow Laboratory of Ocean Sciences and grown in f/2 medium with 12:12 h photoperiod at 24° C.

Cloning and sequencing. The smallest nucleomorph chromosome, called chromosome III (95 kb), was excised from a pulsed field gel (see Fig. 3A for electrophoretic conditions) and the DNA purified with a DNA purification matrix (Prep-a-Gene, Bio-Rad). Chromosomal DNA was treated with T4 DNA polymerase in the presence of dNTPs following the manufacturer's protocols (Promega) to "polish" the ends. The chromosomal DNA was then divided into aliquots and digested with different restriction endonucleases and ligated into suitably prepared plasmid vectors (pGemSZF (→) Promega) with one blunt end and one "sticky" end.

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Bacterial strain XL1-blue (Stratagene) was transformed with the recombinant plastid DNA and white recombinant colonies recovered by blue/white selection. Twenty-four recombinants were screened for the presence of telomeres. Any clones whose insert could be released were rejected as they probably contained double sticky-end ligated products that could not be telomers. Only plasmid clones (4 out of 24) in which the insert could not be released by the appropriate restriction enzyme were retained, as these contained an insert ligated with a restriction endonuclease-resistant blunt end, possibly a telomere. These four clones were tested for the presence of telomeres by sequencing on an automated sequencer (Applied Biosystems 373A) using a dye terminator sequencing kit (Applied Biosystems). Sequence data were analysed by BLASTN searches of GenBank.

Southern analysis. Genomic DNA for restriction enzyme analysis was isolated from pulsed field gel plugs (McPadden et al. 1994a) using a DNA purification matrix (Prep-a-Gene, Bio-Rad). DNA was digested with restriction endonucleases (Promega), electrophoresed in 1% agarose, and blotted onto Zetablot (Bio-Rad) under alkaline conditions following the manufacturer’s protocol. Cloned telomeric DNA fragments to be used as probes were labelled with [32P]dCTP (Feinberg and Vogelstein 1983). Hybridisation experiments were performed at high stringency (48 °C) in buffer containing 50% formamide according to the Zetablot manufacturer’s (Bio-Rad) protocol. Membranes were washed according to Sambrook et al. (1989) at high stringency (68 °C, 0.1xSSC, 0.5% SDS), (1xSSC is 0.15 M NaCl, 0.015 M sodium citrate).

Pulsed field gel electrophoresis. Cells for chromosomal DNA for pulsed field gel electrophoresis were prepared as described by Eschbach et al. (1991). Concentrations of cells in plug preparations are indicated in Fig. 3. Pulsed field gel electrophoresis was performed in a CHEF (contour-clamped homogeneous electric

field) DRIII apparatus (Bio-Rad) in standard 1% agarose in 0.5xTBE at 14 °C. Electrophoresis running times, voltages and pulse times are indicated in Fig. 3. Gels were blotted as above.

Construction of plasmid library. DNA from Chromosome III was isolated from pulsed field gel plugs as described above, digested with SpeI and ligated into suitably prepared plasmid vector (pBS SKII (+), Stratagene). Transformation and selection of recombinant clones were performed as above. Plasmid DNA was prepared, slot-blotted onto Zetablot according to the manufacturer’s protocol and probed as above.

Results and discussion

Cloning of telomeres from nucleomorph chromosomes

Clone pNMT16 contained a 3.5 kb insert with a telomere-like sequence (32 repeats of the 7 base motif TCTAGGG) at one end (Fig. 2B). This guanine-rich repeat is similar to telomeres characterised from other eukaryotes, especially those of angiosperms (TTTAGGG) and the parasitic protozoan Plasmodium (TTT/CAGGG) (Wu and Tanksley 1993; Greider et al. 1993), which differ by a single substitution. The guanine-rich strand in clone pNMT16 was oriented 5' to 3' towards the end of the clone thought to correspond to the nucleomorph chromosome terminus. This orientation is typical of telomere DNA, where the guanine-rich strand creates a short 3' protrusion (Richards et al. 1993).

Sequence from the other end of the insert, which was cloned via a PstI site, was submitted to a database search
that indicated very close matches with 5.8S rRNA gene sequences (Fig. 2B). The orientation of the 5.8S rRNA gene suggested that clone pNMT16 should also contain an srRNA gene if nucleomorph chromosomes adhered to the standard eukaryotic pattern of organisation for rRNA genes. Sequencing confirmed the presence of an srRNA gene (Fig. 2B) of the type previously determined by in situ hybridisation to derive from nucleomorph (McFadden et al. 1994a). This demonstrates that clone pNMT16 containing the putative telomere is of nucleomorph origin.

Telomeric DNA in the nucleomorph

To confirm that the TCTAGGG repeats in clone pNMT16 were from the chromosome terminus, and not an internal fragment, we undertook Southern analysis. If pNMT16 truly represents the end of the chromosome, then probing restriction digests with the telomere motif should create fragments whose size would be predictable. This would even be true for enzymes for which only one recognition site occurred in pNMT16, since the other end of the restriction fragment would correspond to the putative terminus, not a second restriction site. Southern blots of total cell DNA digested with restriction enzymes recognising sites in pNMT16, were probed with an SpeI subclone containing the putative telomere sequence (pNMTelo, GenBank Acc. No. 021303, see Fig. 2C). For the four enzymes tested, the size of labelled fragments was exactly consistent with our hypothesis that the TCTAGGG repeats at one end of pNMT16 are representative of the chromosome terminus (compare the fragment sizes with the restriction map of pNMT16 in Fig. 2).

Interestingly, the smaller restriction fragments (EcoRI and SpeI digests) are heterodisperse in size. Our clone of the terminus contained 32 repeat motifs, but the number of telomeric repeats at the ends of eukaryotic chromosomes is known to be variable (Zakian 1989) and even the number of repeats on an individual chromosome does not remain constant throughout the cell cycle. The heterodisperse size of the smaller fragments (Fig. 2A) is thus highly characteristic of telomeres (Zakian 1989) and suggests that the numbers of repeats on the nucleomorph chromosome is also variable. From the size distribution of the smaller restriction fragments, we calculate that the nucleomorph chromosome carries between 25 and 45 copies of the TCTAGGG repeat at the terminus. Similar numbers of repeat motifs are observed on certain fungal chromosomes (Schechterman 1990).

A second band, 100 bp shorter than the larger band but several fold less abundant, was present in each digest (Fig. 2A). We believe the presence of the second
smaller band, evident in BssHII, EcoRI and most importantly SspI digests, does not indicate heterogeneity within subtelomeric DNA among the ends of the nucleomorph chromosomes. As most of the DNA between the end of the chromosome and the first SspI site (Fig. 2B) consists of telomeric repeats, then it is likely that the smaller band simply contains fewer telomeric repeats than the larger band. It follows that the numbers of telomeric repeats on nucleomorph chromosomes fall into two size classes with the larger size class more common. The smaller band would contain around 18 repeats.

While the Southern analysis confirms that the TCTAGGG repeats of pNMT16 are derived from a chromosome terminus, the labelling of single restriction fragments in each digest appears incongruous. Since we had previously demonstrated that the nucleomorph contained three chromosomes (McFadden et al. 1994a), we had anticipated that probing with a telomere clone would label at least six fragments rather than just one. The labelling of a single restriction fragment with the TCTAGGG repeat could be interpreted in two ways: either we had cloned a terminus unique to one end of nucleomorph chromosome III, or all nucleomorph chromosome termini are identical with respect to the restriction sites examined. Since sequencing of pNMT16 had revealed an rRNA gene unit located very close to at least one terminus of chromosome III, and previous investigations had demonstrated that chromosomes I and II also carry rRNA genes (McFadden et al. 1994a), the presence of rRNA genes associated with the telomere in an identical manner at the termini of the other chromosomes seemed a distinct possibility. Further experimentation confirmed this.

**Nucleomorph karyotype and telomeres**

We anticipated that if pNMT16 contained a genuine telomere it would hybridise not only to chromosome III but to all nucleomorph chromosomes. When the subclone (pNMTelo) containing 32 repeats of the TCTAGGG motif was hybridised to pulsed field gel blots, three chromosomes – which had previously been allocated to the nucleomorph using the nucleomorph-specific srRNA (McFadden et al. 1994a) – were labelled (Fig. 3A, lane 4, and 3B, lane 4). This result has several implications. Firstly, it demonstrates that three different chromosomes carry the telomere motif. Since, Southern analysis of restriction digests with the TCTAGGG motif labels only one fragment, all six ends of these chromosomes must be identical, and most probably have rRNA gene units located near the telomeres as depicted in Fig. 2B. There are apparently no internal telomeric sequences or more than one fragment would be labelled in each digest. Finally, probing with the telomere motif, an element presumed to be carried by all chromosomes present in the nucleomorph, reveals the presence of only three chromosomes. This indicates a haploid genome size of only 380 kb for the nucleomorph. However, without having isolated the chlorarachniophyte nucleomorph we cannot be absolutely certain that it does not contain oth-

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**Fig. 3A, B.** Southern analyses of chlorarachniophyte chromosomal DNAs with telomere probes. A DNA was electrophoresed as follows: pulse time 100 s at 100 V for 3 h followed by a pulse time ramped from 60-120 s at 200 V for 36 h in 0.5xTBE and 1% agarose. Lanes 1 and 2, ethidium bromide stained gel of *Saccharomyces cerevisiae* (Bio-Rad) and chlorarachniophyte isolate CCMP 621 chromosomes (1.8x10⁸ cells/ml). Lanes 3 and 4, an autoradiograph of a Southern blot of lanes 1 and 2 probed with pNMTelo. Lanes 3 and 5, an autoradiograph of a Southern blot of lanes 1 and 2 probed with pNla17. B DNA was electrophoresed with a pulse time of 20 s for 16 h and 10 s for 16 h at 175 V in 0.5xTBE and 1% agarose. Lanes 1 and 2, ethidium bromide stained gel of Lambda concatamers (Bio-Rad) and chlorarachniophyte isolate CCMP 621 chromosomes (4.3x10⁹ cells/ml). Lanes 3 and 4, an autoradiograph of a Southern blot of lanes 1 and 2 probed with pNMTelo. Lanes 5 and 6, an autoradiograph of a Southern blot of lanes 1 and 2 probed with pNla17. Species names and the repeat motif of each probe are indicated above each lane. Chromosome sizes markers are in kb (A, B, lane 1) and nucleomorph chromosomes I, II and III are shown (A, B, lane 2).
er chromosomes, which for reasons unknown, lack TCTAGGG telomeres.

In cryptomonad algae (which like chlorarachniophytes contain a eukaryotic endosymbiont with nucleomorph) physical isolation of the nucleomorph (Hansmaan and Eschbach 1990) has allowed karyotyping by pulsed field gel electrophoresis (Eschbach et al. 1991). Intriguingly, the cryptomonad nucleomorph also contains three small chromosomes with a haploid genome size between 550 and 660 kb (Eschbach et al. 1991; McFadden et al. 1994b). In cryptomonads the three nucleomorph chromosomes also encode rRNA genes, but the termini have not been characterised (Eschbach et al. 1991; McFadden et al. 1994b) so it is not yet possible to fathom the similarity in karyotypes of the vestigial endosymbiont nuclei in these two organisms.

**Nuclear karyotype and telomeres**

Since the TCTAGGG telomere was exclusive to nucleomorph chromosomes, we decided to determine what telomere motif was present on the nuclear (host cell) chromosomes. We probed pulsed field gel blots of separated chlorarachniophyte chromosomes with pNla17, a clone of telomeric and subtelomeric DNA from the ascomycete *Fusarium oxysporum*, containing 17 copies of the 6 bp motif (TCTAGGG) (Powell and Kistler 1990). This probe was chosen because it contains a common telomeric motif occurring in vertebrates, some fungi and several protists (Zakian 1989). The TCTAGGG bound at high stringency to all chromosomes of 325 kb or larger (Fig. 3A, B, lane 6). In chlorarachniophyte strain CCMP 621 there are about 16 chromosomes ranging in size from 325–2000 kb. Only chromosomes smaller than 1400 kb are resolved in Fig. 3A, lane 2. Because some of the chromosomes that bind pNla17 were previously identified as coming from the host nucleus (McFadden et al. 1994a), it is very likely that all pNla17-binding chromosomes are derived from the host nucleus.

Although the TCTAGGG motif showed weak labelling to the three nucleomorph chromosomes, it did not label the chromosomes of *Saccharomyces cerevisiae* used as markers (Fig. 3A, lane 3). We anticipated yeast chromosomes would not label as the *S. cerevisiae* telomeric sequence [(TG)₃]₉ (Shampay et al. 1984) is quite different from that of the probe. The weak labelling of nucleomorph chromosomes by the TCTAGGG is probably attributable to cross-hybridisation between slightly different telomeres, as has been observed between *Arabidopsis* (TCTAGGG) and human (TCTAGGG) telomeres (Richards and Ausubel 1988). Based on our hybridisation data we tentatively assign the nuclear telomeric repeat as TCTAGGG.

The only chlorarachniophyte chromosomes unaccounted for by either of the two telomeric probes were

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**Fig. 4.** Map of nucleomorph chromosome III. Chromosome III (centre) terminates in inverted repeats which have been expanded to show gene and telomere detail. Part of the inverted repeat consists of pNMT16 which could be derived from either end of chromosome III and contains a telomere linked to part of the rRNA cistron. Overlapping with pNMT16 are the 11.7 kb (top) and 13.2 kb SpeI clones (bottom). These clones contain the remainder of the inverted repeat with a single near complete rRNA cistron. The unique regions of the SpeI clones begin at arrows. Restriction sites are indicated as follows: E EcoRI, H HindIII, P PstI, S SpeI.
the two smallest chromosomes of sizes 36 and 72 kb (Fig. 3). We believe that these two chromosomes are not part of either the nucleomorph or nuclear genomes but represent the mitochondrial genome. Cloning and sequencing of fragments from these chromosomes has revealed genes for enzymes in the respiratory pathway (Gilson unpublished) suggesting that they are mitochondrial chromosomes. Although mitochondrial chromosomes are typically circular in vertebrates and plants, linear mitochondrial genomes are common in protists (Bendich 1993). The migratory characteristics of the chlorarachniophyte mitochondrial chromosomes in a pulsed field gel suggest they are also linear. Linear mitochondrial chromosomes can carry repetitive terminal sequences (Vahrenholz et al. 1993) but as they are unlike nuclear chromosomal telomeric repeats they are not expected to bind with the TTAGGG or TCTAGGG repeat motifs used in this study.

Mapping of nucleomorph chromosome III

We have determined that each nucleomorph chromosome terminates in identical inverted repeats, each repeat containing a telomere linked to an sRNA/5.8S gene unit (Figs. 2B, 4). To determine whether there was unique sequence between the repeats, we conducted a chromosomal walk from each end of nucleomorph chromosome III towards the interior. Plasmid libraries of chromosome III were probed with a small fragment specific for the nucleomorph sRNA gene (see McFadden et al. 1994a). Two clones (11.7 and 13.2 kb) containing the nucleomorph sRNA gene were recovered from an SpeI library (Fig. 4). To confirm that these were the only SpeI fragments containing sRNA genes, chromosome III was isolated from pulsed field gels and digested with SpeI. Only two SpeI restriction fragments (11.7 and 13.2 kb) were labelled when a Southern blot of the digested chromosome was probed with the nucleomorph-specific sRNA gene probe (data not shown). This indicates that all sRNA genes are confined to these fragments and that chromosome III carries only two copies of the sRNA gene.

Subcloning and partial sequencing of the two large SpeI clones was conducted to determine (1) which rRNA genes were present, (2) were these rRNA genes the same as those found in the telomeric clone pNMT16 and (3) how much unique DNA existed in each clone. Restriction maps of the SpeI clones are presented in Fig. 4. Both SpeI clones overlap clone pNMT16 with perfect identity, further confirming that the termini must be identical. Inwards from the PstI site adjacent to the 5.8S gene that defines one end of pNMT16, we identified a large subunit rRNA (lrRNA) gene in each clone (Fig. 4). The nucleomorph rRNA genes are thus arranged in the manner standard to most eukaryotes. Organisation of the rRNA genes is probably similar on the other two nucleomorph chromosomes since probing of chromosomes I and II with a fragment from the 3' end of the lrRNA gene (subcloned from the 13.2 kb SpeI clone of chromosome III) demonstrated that lrRNA genes are also present on these two chromosomes (data not shown). Considering the conservative nature of rRNA gene repeats, the rRNA gene transcription units of the chromosomes I and II are very likely identical to chromosome III.

Comparison of the restriction maps for the 11.7 and 13.2 kb SpeI clones indicated that inwards from the HindIII site adjacent to the 3' end of the lrRNA gene, the pattern of restriction sites differed in the two clones (Fig. 4). Sequencing showed that the two clones become different at a point approximately 550 bp inwards of this HindIII site (arrows in Fig. 4). Beyond this point the restriction maps and sequence show no similarity thus far. Chromosome III thus comprises 75–80 kb of apparently unique DNA bordered by inverted, identical 8.5 kb terminal repeats containing an rRNA cistron (Fig. 4).

Based on preliminary analysis of the unique region within chromosome III, we estimate the nucleomorph could potentially encode about 100–150 genes (not including rRNA genes). Clearly this gene complement is insufficient for autonomy, and the endosymbiont must be dependent on the host for numerous products. The current challenge is to determine which genes remain in this curious organelle and why. Since the nuclear and nucleomorph telomeres differ, telomerase genes of the endosymbiont are presumably different from those of the host and would not have been rendered redundant upon entering an endosymbiotic partnership. It therefore seems likely that the nucleomorph will encode its own telomere-producing components. The small size of the nucleomorph genome should facilitate characterisation of these and other genes that have been retained.

The terminal inverted repeat arrangement of nucleomorph chromosomes is somewhat reminiscent of chloroplast chromosomes in which a small region of single copy DNA is separated from a larger region of single copy DNA by two inverted rRNA transcription units (for reviews see Howe et al. 1992; Palmer 1992; Ohyama 1992). The inverted repeats of chloroplast genomes apparently enhance chloroplast genome stability through intramolecular recombination (Boynton et al. 1992; Palmer 1992) and serve to keep genes within the inverted repeat identical (Reith and Munholland 1993). The termini of nucleomorph chromosomes would also appear to be kept identical by crossing over, but in this case the chromosome is linear not circular. Identity of all nucleomorph chromosome ends implies that both intra- and intermolecular crossing over occurs. This is congruent with the observation that telomeric regions of other eukaryotic chromosomes possess repetitive elements and are recombinational hot-spots (Charron et al. 1989; De Bruin et al. 1994). Apart from a perceived need to keep all copies of rRNA genes identical, the reason why a single rRNA gene transcription unit should be linked to each telomere is unclear. rRNA gene repeats are linked to telomeres in the primitive eukaryote Giardia lamblia (Le Blancq et al. 1991), and nucleomorph gene organisation is perhaps a hangover of some primitive type of chromosome organisation.
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