Plastid-Derived Single Gene Minicircles of the Dinoflagellate 
*Ceratium horridum* Are Localized in the Nucleus

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Recent reports show that numerous chloroplast-specific proteins of peridinin-containing dinoflagellates are encoded on minicircles—small plasmidlike molecules containing one or two polypeptide genes each. The genes for these polypeptides are chloroplast specific because their homologs from other photosynthetic eukaryotes are exclusively encoded in the chloroplast genome. Here, we report the isolation, sequencing, and subcellular localization of minicircles from the peridinin-containing dinoflagellate *Ceratium horridum*. The *C. horridum* minicircles are organized in the same manner as in other peridinin-containing dinoflagellates and encode the same kinds of plastid-specific proteins, as previous studies reported. However, intact plastids isolated from *C. horridum* do not contain minicircles, nor do they contain DNA that hybridizes to minicircle-specific probes. Rather, *C. horridum* minicircles are localized in the nucleus as shown by cell fractionation, Southern hybridization, and in situ hybridization with minicircle-specific probes. A high-molecular-weight DNA was detected in purified *C. horridum* plastids, but it is apparently not minicircular in organization, as hybridization with a cloned probe from the plastid-localized DNA suggests. The distinction between *C. horridum* and other peridinin-containing dinoflagellates at the level of their minicircle localization is paralleled by *C. horridum* thylakoid organization, which also differs from that of other peridinin-containing dinoflagellates, indicating that a hitherto underestimated diversity of minicircle DNA localization and thylakoid organization exists across various dinoflagellate groups.

Introduction

The organization of the genetic material in plastids of peridinin-containing dinoflagellates, a group of photosynthetic, unicellular alveolate eukaryotes, is thought to be unique in that a minor portion of chloroplast proteins and ribosomal RNAs appear to be individually encoded on plasmidlike “minicircles” of approximately 2 to 3 kb in size (Zhang, Green, and Cavalier-Smith 1999; Barbrook and Howe 2000; Zhang, Cavalier-Smith, and Green 2001; Barbrook et al. 2001; Hiller 2001), rather than collectively on evolutionarily reduced cyanobacterial chromosomes of approximately 100 to 200 kb in length, as in all other photosynthetic organelles (Martin et al. 1998; Douglas 1998; Stoebe and Kowallik 1999). Moreover, recent work shows that gene transfer from the plastid into the cell nucleus is unusual high, making peridinin-containing dinoflagellates the “champions” in this field (Hackett et al. 2004).

Sequence similarities indicate that minicircle-encoded genes are of cyanobacterial origin (Zhang, Green, and Cavalier-Smith 1999), and it is generally assumed that these unusual molecules reside in the plastid of the species studied so far (Zhang, Green, and Cavalier-Smith 1999; McFadden 1999; Barbrook et al. 2001). However, in none of those pioneering studies on dinoflagellate minicircles has any direct evidence been reported that the minicircle DNA itself might be plastid. Recent studies have shown that minicircle mRNA transcripts (though not minicircle DNA) are indeed found in the plastid of *Symbiodinium* (Takishita et al. 2003), providing additional indirect evidence that the minicircle DNA itself might be plastid localized in the peridinin-containing dinoflagellate *Symbio-

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Key words: Dinoflagellates, plastids, minicircles, peridinin.

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doi:10.1093/molbev/msi127
Advance Access publication March 19, 2004

Isolation of *C. horridum* Chloroplasts

*C. horridum* (100 to 200 mg) was resuspended in an ice-cold isolation buffer (0.4M mannitol, 1mM MgCl₂, 0.1M Tris-HCl, 0.5mM EDTA, 10mM MgCl₂, and 0.5mM dithiothreitol) and homogenized using a teflon pestle. Minicircles were isolated by following the a Li-Cor 4200 sequencer and sequences thus obtained

deposited at SAG Göttingen (www.gwdg.de/~epsag/phykologia/epsag.html).
10mM Na₂EDTA, 0.25% polyvinylpyrrolidone 90, 50mM Tris-HCl pH 7.5), homogenized in a 5-ml potter and filtered through a 5-μm mesh gauze. The chloroplasts were pelleted from the filtrate at 3,400 g, resuspended in a 150-μl isolation buffer, loaded on 30% (v/v) Percoll (Amersham) in isolation buffer and centrifuged at 4,000 g for 10 min.

**Isolation of C. horridum Nuclei**

*C. horridum* cells were suspended and homogenized as for plastid preparation (mannitol concentration was increased to 0.7 M). The homogenate was filtered through a 22-μm mesh gauze and centrifuged at 2,500 g to 3,000 g. The pellet was resuspended and loaded on 30% to 40% (v/v) Percoll (Amersham). After centrifugation for 10 min and 3,400 g the gradient was fractionated, and fractions containing intact nuclei were identified microscopically.

**Electron Microscopy**

Ultrathin sections of trypsin-treated *Prorocentrum micans* were prepared as described (Kowallik 1971) and recorded in a Philips EM 301 electron microscope as described (Herrmann et al. 1975).

**In Situ Hybridization**

In situ hybridizations were performed as described (McFadden 1991). The probes used for hybridizations were a 1,244-bp biotinylated probe spanning the coding region of the psbB minicircle, a 1,038-bp probe from the RuBisCO gene of *C. horridum*, and a 64-bp (5′-CCAGTGCTGGTTGTTGTTGTTGTTGCGCCGGCTGTTGTTGTTGGG-3′) fragment cloned from the high molecular plastid DNA from *C. horridum*. As a negative control, in situ hybridizations were done with the secondary antibody only. Hybrids were immunodetected for electron microscopy with gold-coupled antibiotin antibodies and with Cy2-coupled antibiotin antibodies for confocal laser scanning microscopy.

**Results and Discussion**

Minicircles from *Ceratium horridum* were enriched from total dinoflagellate DNA by a procedure that exploits their plasmid-like size and conformation (figure 1, lane B). The most prominent circular minicircle size populations in *C. horridum* migrate in the range of 3.5 to 4 kb in agarose gels (figure 1, lane B), similar to the size range of cloned minicircles previously found for *Heterocapsa triquetra*, *Amphidinium operculatum*, and *Amphidinium carterae* (Zhang, Green, and Cavalier-Smith; Barbrook and Howe 2000; Zhang et al. 2001; Barbrook et al. 2001; Hiller 2001).

Sequence comparisons showed that the *C. horridum* minicircles encode genes of cyanobacterial origin, homologs of which are plastome-localized in other algae. By cloning of isolated minicircles, we detected genes and gene fragments for plastid-related *psaA*, *psaB*, *psbB*, *psbC*, *psbD*, *psbE*, *petB*, *ycf16*, and *ycf24*. Whereas the genes *psaA*, *psaB*, *psbB*, *psbC*, *psbD*, *psbE*, and *petB* are contained in all plastid genomes sequenced to date, *ycf16* and *ycf24* are specific to plastid genomes from non–green algal lineages (Stoebe, Martin, and Kowallik. 1999). All minicircle-encoded proteins from peridinin-containing dinoflagellates isolated here and previously (Zhang, Green, and Cavalier-Smith 1999; Barbrook and Howe 2000; Zhang, Cavalier-Smith, and Green; Barbrook et al. 2001;...
Fig. 2.—In situ hybridizations. (a) and (e) show phase contrast images of (b) and (f), respectively. In situ hybridizations are shown in (b) using a plastid genome–derived probe and a psbB probe in (e). (c) Visualizes a mechanically stressed C. horridum cell releasing the nucleus and plastids. These organelles (P = plastid; N = nucleus), before its separation, are shown in (d). (g) Hybridization using the minicircle-encoded psbB coding region of C. horridum as probe and visualizing the hybrids with the electron microscope. Numerous hybrids in the cell nucleus (N) and one in the plastid (P) are shown by arrows. The bar = 1 μm.

Hiller 2001) share convincing amino acid similarity with their homologs from red algal chloroplast genomes (and plastids derived from red algae via secondary endosymbiosis). A significant portion of minicircles from C. horridum encode one protein per plasmid, except ycf16/ycf24, which are located on a single minicircle in tandem. As with other dinoflagellates (Barbrook et al. 2001; Hiller 2001), several C. horridum minicircles did not contain a recognizable coding region. We found no typical transit peptide coding regions contiguous with the minicircle open reading frames, consistent with findings from previous studies.

To physically localize the minicircles within the cell, intact plastids were purified from C. horridum by cell fractionation, and their nucleic acids were isolated. Purified plastids did not contain detectable amounts of minicircle DNA (figure 1, lanes D and F), whereas DNA from whole cells did (figure 1, lane C). This was confirmed by Southern hybridization with a minicircle-specific probe (figure 1, lanes C* to G*). Instead, the plastids release a high-molecular-weight DNA upon lysis (figure 1, lanes D and F). To further pinpoint the localization of these minicircles, we isolated nuclei from C. horridum. Minicircles can be observed in large numbers in the nuclear DNA (figure 1, lane H), which was confirmed by Southern hybridizations with a minicircle probe (figure 1, lane H*).

We tried to demonstrate the presence of minicircles in the dinoflagellate plastids by in situ hybridization and imaging with confocal laser scanning microscopy, using a 1,244 bp long stretch from the coding region of minicircle psbB from C. horridum (fig. 2f) as well as a fragment from high-molecular-weight plastid DNA (fig. 2b) as probes. An additional control was performed in which the secondary antibody was tested for unspecific reactions (not shown), showing the autofluorescence of the plastids. As shown in figure 2f, a minicircle probe labeled the nucleus in the space between the chromosomes. Furthermore, the high-molecular-weight plastid probe hybridized in the plastid (fig. 2b).

That the psbB probe specifically labeled the interchromosomal regions of the nucleus was confirmed by conventional in situ hybridization techniques and visualization with electron microscopy (fig. 2g). As a further control, a probe spanning the C. horridum–specific RuBisCo labeled a chromosomal region, whereas a nonhomologous probe (phycoerythrin from cryptomonads) showed no significant hybridization (data not shown). Taken together, these findings indicate that the sequence of the psbB minicircle is primarily, and probably exclusively, localized extrachromosomal in the nucleus and not in the plastid of C. horridum.

In a recent study, minicircle-encoded mRNA for psbA was detected in the chloroplast of the genus Symbiodinium by in-situ hybridization (Takishita et al. 2003). These data, together with the results of Zhang, Green, and Cavalier-Smith (1999) on Prolocentrum, from which no 23S rRNA was detected, raises the possibility that in some dinoflagellate species, minicircles may be plastid located, whereas in others, the chloroplast genes have moved into the cell nucleus (Cavalier-Smith 2003). This hypothesis is complicated by the finding that in some dinoflagellate species, plastid DNAs have been identified with molecular sizes between 114 to 125 kb, consistent with plastid DNAs from other phototrophic organisms (Boczar, Liston, and Catollico 1991).

This conundrum of findings prompted us to start again from scratch to identify the genuine dinoflagellate plastid DNA from C. horridum. A plastid-localized DNA can be clearly seen inside the dinoflagellate stroma by traditional transmission electron microscopy of, for example, Prolocentrum micans (fig. 3). To find the plastid DNA in Ceratium, intact plastids were isolated from C. horridum. Isolated plastids that were lysed after treatment with DNase
I released a high-molecular-weight DNA that is DNase sensitive (figure 1, lane K). C. horridum minicircles do not hybridize to the high-molecular-weight DNA isolated from such purified plastids (figure 1, lane K+), but do hybridize to the supernatant of this preparation, which contains broken nuclei (figure 1, lanes L and L+). Thus, the C. horridum plastid DNA is apparently distinct from minicircle DNA. However, in Southern experiments, a probe cloned from the plastid DNA fraction (see Methods) hybridized with the high-molecular-plastid DNA (figure 1, lane K*), as well as with the DNA isolated from the supernatant of the plastid preparation, which contains DNA from the other organelles and from broken plastids (figure 1, lane L*). Finally, in situ hybridization experiments with the probe for the high-molecular-weight DNA confirmed its plastid location (fig. 2b). Nevertheless, the chloroplast-located DNA of C. horridum remains mysterious. Sequences obtained from this genome do not show any coding region or significant similarities to other data base entries.

Peridinin-containing dinoflagellates are clearly defined by their plastid morphology: a triple membrane envelope and thylakoid stacks in layers of three. Because our findings suggested that there may be lineage-specific differences of minicircle localization among peridinin-containing dinoflagellates, we reinspected the plastid morphotype of C. horridum. One of us (KVK) noticed that differences are obvious, because the C. horridum plastid shows stacks of thylakoids in rows of two in contrast to the stacks of three typical for other peridinin-containing dinoflagellates. Furthermore, the thylakoid lumen is opaque as in cryptophyte plastids. However, the plastids are devoid of phycobiliproteins but contain peridinin.

Our findings indicate that the organization of the dinoflagellate C. horridum minicircles is surprising because it is a nuclear extrachromosomal and not a plastid genome organization, although in evolutionary terms, it ultimately stems from the three-membrane-bounded plastid of these protists. DNA does exist in plastids from C. horridum, but it is distinct from the nuclear minicircles previously thought to exclusively represent the chloroplast DNA.

Where are plastid proteins expressed? If they are expressed in the cytoplasm of the host, how do they find their final target sites? If the minicircles of C. horridum (but possibly not those from other dinoflagellates [Takishita et al. 2003]) represent only vehicles for transferring plastid DNA into the cell nucleus, one would have to postulate that the plastid-located DNA does indeed encode a set of proteins known from other chloroplasts (Martin et al. 2002). Our preliminary analysis of the plastid genome shows yet no indications of such encoded genes. However, coding regions could be hidden and recognized only after an editing machinery, known from chloroplasts of higher plants (Bock 2000), has formed an mRNA that could be reasonably translated. If minicircles are the transportation molecules into the nucleus, one would suggest that dinoflagellates show an extended rate of plastid gene migration into the host. Hints for this comes from a recent publication showing that 15 genes that are limited to the plastid genome in all other eukaryotic phototrophs are nucleus encoded in a peridinin-containing dinoflagellate (Hackett et al. 2004).

As most of the minicircle-encoded proteins are known to be expressed in chloroplasts of other groups, one could argue that RNA import might provide the plastid with RNA messages. Such a roughly analogous situation is known, for example, in the case of tRNA import into mitochondria of trypanosomes (Schneider 2001). If the C. horridum nuclear minicircles specify chloroplast proteins, then these would be expected to encode a preprotein, harboring a signal sequence and a transit peptide to manage the transport across the plastid surrounding membranes. However, the coding region does not show any N-terminal extension that could serve as a signal sequence. Again, topogenic signals could be hidden in the genomic sequence and uncovered in the mRNA after RNA editing. Notably, editing can be observed in C. horridum minicircles (Zauner and Maier, unpublished data).

Dinoflagellates are unusual in many respects, as shown, for example, by their chromosome architecture and by the finding that peridinin-containing dinoflagellates express an unusual form II RuBisCo (Morse et al. 1995, Whitney, Shaw, and Yellowlees 1995, Rowan et al. 1996). C. horridum furthermore differs from other phototrophic dinoflagellates investigated thus far by its noncanonical plastid morphology, despite of the existence of peridinin, and by its minicircle localization, assuming that all other dinoflagellate minicircles described thus far are localized in the plastid. Thus, peridinin-containing dinoflagellates could be a heterogenous subgroup of alveolates, in which nature might have found a genetic playground.

Supplementary Material

Sequences are deposited in GenBank under accession numbers AF490356 to AF490364 and AF490368.

Acknowledgments

The authors would like to thank Marianne Johannsen (Marburg) and Dr. Franz Grolig (Marburg) for their help in electron and confocal laser scanning microscopy. Supported by the Deutsche Forschungsgemeinschaft.

Literature Cited


William Martin, Associate Editor

Accepted February 27, 2004