Introduction

The hypothetical origin of mitochondria and chloroplasts as bacterial endosymbionts was supported by evidence that these organelles contained their own genomes, similar to those of prokaryotes [7]. That the organelles’ proteins are more similar to bacterial proteins than to other proteins in the same cell helped confirm the idea of an exogenous origin [20]. The symbiogenetic origin of the eukaryotic flagellum or undulipodium from a spirochete bacterium was further hypothesized by Lynn Margulis, formerly L. Sagan [20, 27]. This hypothesis can be tested by looking for eukaryotic protein homologues, such as tubulin, in spirochetes or by examining extant protists to see if they contain spirochete genes in their nuclei or have DNA of spirochete origin in or associated with their kinetosomes in a manner analogous to the genomes of mitochondria and chloroplasts. Although cytoplasmic tubules have been observed in spirochetes and other groups of bacteria [1], no tubulin homologous protein has been found in spirochetes. A putative prokaryotic tubulin homologue, FtsZ [5], is widespread among eubacteria and archaeabacteria.

The search for kinetosome DNA has focused on more modern organisms such as green algae, plants and ciliates. Molecular systematic studies identify early branching eukaryotic lineages (diplobionts, trichomonads [29]) that are amitochondriate and are characterized by a cytoskeleton organized around a karyomastigont, an undulipodial system associated with a nucleus, and other structures, including a parabasal body or Golgi complex in the case of trichomonads. In some of these lineages the mastigonts are disassociated from the nuclei and are called akaryomastigonts.

The multimastigont trichomonad, Snyderella tabogae, is an ideal cell to test for kinetosome DNA because it is 100 μm long and its cortex is covered by over a thousand kinetosomes arranged in discrete sets of four, the akaryomastigonts [4]. In contrast to other archaeprotists, its multiple nuclei are separated from its kinetosomes. Therefore, if DNA could be visualized associated with the peripherally situated centriole-kinetosomes (c-k’s), the distinctive surface pattern...
that resulted could not be confused with other organelles. As representatives of an early branching lineage, trichomonads may retain ancestral characters that are lost from more recently evolved, derived forms. Although Snyderella is from the early branching trichomonad lineage, it evolved within the last 100 million years as a symbiont in the hindguts of termites.

Reports of DNA in or associated with the c-k have been controversial for many years. Most tests to find c-k DNA have been negative. Feulgen reaction tests for DNA in the blepharoplasts of plant spermatogenous cells, which generate c-k’s de novo, have been negative for the fern Marsilea and the cycad Zamia [21], and for nine different bryophytes and ferns [33]. The DAPI stain was negative for Marsilea blepharoplasts [22]. Unspecified negative results were reported for Ceratopteris blepharoplasts [13]. The only positive result, using the Feulgen stain on Ginkgo [19], was not supported in a subsequent study [6].

Studies which reported RNA in, or associated with c-k’s, found no evidence for DNA associated with these same structures. While RNase removed pericentriolar material in PTK2 cells (rat kangaroo kidney cells), DNase had no effect on the c-k structure [23]. Isolated Chlamydomonas and Tetrahymena c-k’s treated with RNase were prevented from nucleating asters when injected into Xenopus eggs, but DNase-treated c-k’s functioned normally [11]. RNase-treatment modified Paramecium c-k structure, but DNase-treatment did not [3]. Acridine orange-staining material in Tetrahymena c-k’s was removed by RNase-treatment, but was not removed by DNase [10]. Other studies which found no evidence for RNA in the c-k [12, 16, 17, 25] also found no evidence for DNA there.

Two reports of DNA in ciliate c-k’s in Tetrahymena [24], and in Paramecium [28] were not supported by subsequent studies [10] in Tetrahymena, and [30] in Paramecium. Because mitochondria are aligned at ciliary bases the false-positive detection of c-k DNA in ciliates are most likely attributable to the presence of regularly-patterned mitochondrial DNA [32]. DNA was reported in the centriole-kinetosomes of Chlamydomonas reinhardtii [8]. As these findings could not be confirmed by immunocytochemistry [15], DAPI staining [18] and genetic analysis [14], further experiments documented that the DNA sequence of the earlier study is in the nucleolus and is positioned adjacent to the centriole-kinetosomes in interphase [9].

The fluorescent DNA stains acridine orange, DAPI, ethidium bromide, propidium iodide and SYTOX and the brightfield Feulgen stain were used in this study to test for DNA in the kinetosomes of S. tabogae, Stephanonympha sp. and Caduceia versatilis. While these cells lack mitochondria, they all have characteristic sets of bacterial symbionts. S. tabogae tends to be covered with small coccoid bacteria. Large rods and spirochetes attach to its posterior. In Stephanonympha sp. bacteria are regularly positioned within the axostyle adjacent to each of its nuclei. Intranuclear bacterial symbionts also are present. C. versatilis has at least five distinct bacterial symbionts: two epibiotic, two cytoplasmic and one intranuclear [2].

Materials and methods

Cryptoter mes cavifrons was obtained from southern Florida. It contains three large trichomonad species: C. versatilis, Stephanonympha sp., and S. tabogae. Termites were kept in glass Petri plates with filter paper as a food source. A drop of distilled water was added to the paper every other day. The filter paper had been saturated in a solution of 10,000 U penicillin and 10 mg streptomycin per ml in 0.9 % NaCl to remove surface bacteria from the cells. Termites were reared on this substrate for at least two weeks before they were sacrificed and their gut contents were broken open in a few drops of Trager’s solution [31]. Gut samples from termites that were not fed antibiotics were also used. Cells were pipetted into Eppendorf tubes containing 1 ml of 1% glutaraldehyde in water. Other fixation mixtures contained 0.2% Triton X-100. The hindgut protists of Incisitermes nr. incisus and Incisitermes snyderi were also examined and stained.

The fixed samples were centrifuged at 9 xg and washed once in 0.1 M phosphate buffered saline (PSA) and then stained in one of the following solutions for 30 min: 1 µM acridine orange, 2 µM DAPI (4,6-diamidino-2-phenylindole, Sigma, St. Louis, MO), 2 µM ethidium bromide, 2 µM of propidium iodide, 1 µM SYTOX (Molecular Probes, Eugene, OR) or 1 µM DIOC7 (3,3-diheptyloxacarbocyanine iodide, Sigma). Cells were washed once with 0.1 M PSA, allowed to settle to the bottom of the tube and then pipetted onto a microscope slide as a wet mount. Coverslips were sealed with fingernail polish. Slides were examined under a Nikon epifluorescence microscope. Photographs were taken with Kodak 160 Tungsten color slide film. Video tape was recorded using a CCD camera and 3/4 inch tapes on a Sony U-matic tape deck.

DNase-treatment Cells were treated with DNase I following an amended procedure of Dipple [3]. Specimens were fixed and washed as above, and then exposed to 1.0–2.0 mg/ml DNase I in 0.001 M PSA containing 0.01M MgSO4·7H2O at pH 6.6 for 6 hours at 37°C. Cells were then centrifuged and washed in 0.1 M PSA and placed in 5% trichloroacetic acid (TCA) for one hour at 4°C. The specimens were then washed again and prepared as a wet mount. Cells were squashed by gentle pressure on the coverslip with a dissecting needle.

Feulgen stain The termite hindguts were extracted and broken open in a small drop of Trager’s solution on a poly-l-lysine-coated coverslip. The gut contents were gently smeared on the coverslip, which was then submerged in a Columbia jar filled...
with either 1% glutaraldehyde, Bouin’s fixative (picric acid in formalin) or 70% ethanol and fixed for one hour. These coverslips were washed in 0.1 M PSA and then placed in 1 N HCl at 60°C for 5 min. They were then dipped in 1 N HCl at room temperature for one minute and placed in a decolorized basic fuchsin solution for 30 min. The coverslips were removed and placed in three consecutive solutions of potassium metabisulfite (5 ml of 10% aqueous $K_2S_2O_5$ with 5 ml 1 N HCl and 100 ml distilled water). They were then washed in water and dehydrated in a series of ethanol and mounted with Permount (Fisher, Fairlawn, NJ).

**Results**

**Fluorescence microscopy** A consistent pattern of staining that coincided with the placement of kinetosomes at the cell cortex was sought. *Snyderella tabogae* contains hundreds of kinetosomes in groups of four over most of its cortex. In all cases, the SYTOX-stained cells lacked any fluorescence in the akaryomastigonts in preparations in which bacteria and nuclei, as expected, were well stained for DNA. Conspicuous fluorescence of the akaryomastigonts was detected in DAPI-, acridine orange-, ethidium bromide- and propidium iodide-stained cells. The clearest and most specific staining of nuclei and akaryomastigonts occurred with DAPI preparations (Fig. 1). DAPI fluorescence was not found at the level of the kinetosomes, but just below it (Fig. 2). The same pattern of fluorescence was observed in cells stained with DIOC$_7$, which binds to lipid-rich structures such as the Golgi complex.

While it was impossible to remove all the nuclear DNA as detected by DAPI, the use of 2.0 mg/ml DNase conspicuously reduced nuclear fluorescence. The enzyme treatment was therefore effective in removing some DNA. The extraction of DNA by DNase requires treatment with cold 5% TCA. After DAPI staining, fluorescence was absent from the DNase-treated akaryomastigonts. However, in DAPI-stained controls lacking DNase but treated for one hour with 5% TCA the akaryomastigonts also did not fluoresce.

The akaryomastigonts of the cells that were fixed with the detergent Triton X-100 in the fixative mixture did not fluorescence with DAPI or any of the other DNA stains (acridine orange, ethidium bromide and propidium iodide). Examination of *Caduceia versatilis*, which has a large parabasal body that coils around the base of its nucleus, showed that the detergent had dissolved some of the parabasal cisternae. Examination of non-detergent-treated lysed cells whose mastigont system remained intact showed that DAPI stained the parabasal bodies of *Snyderella, Stephanonympha* and *Caduceia* (Fig. 3).

Varying the DAPI concentration resulted in a pattern of fluorescence of the mastigont component structures (Table 1). Nuclei stained at the lowest concentration, 0.58 μM. The parabasal bodies stained at a higher concentration, 1.45 μM, and the microtubular structures (axonemes and axostyles) stained at the highest concentration, 2.4 μM.

The parabasal body of related calonymphid trichomonads...
(Coronympha octonaria and Metacoronympha senta) in Incisitermes nr. incisus, did not stain with DAPI. Neither was such stain seen in other parabasalids, the hypermastigids, Trichonympha agilis in R. flavipes, and Trichonympha chattoni in I. nr. incisus.

**Feulgen results** Only the nuclei of the three species stained with the Feulgen reagent in cells fixed with picric acid or alcohol. Staining was present in the akaryomastigonts of S. tabogae fixed with glutaraldehyde in some cases. Since the Feulgen reagent binds to aldehyde residues, as in acid-treated DNA, this staining was due apparently to aldehyde residue that was bound to or not washed out of Golgi cisternae in the akaryomastigonts.

**Discussion**

Six different DNA-specific treatments stained the single and multiple nuclei in C. versatilis, Stephanonympha sp. and S. tabogae. Whereas the nucleoids of the regularly associated ecto- and endosymbiotic bacteria stained as well, kinetosomes never stained at all. Neither the DAPI nor the other staining (SYTOX, acridine orange, ethidium bromide, propidium iodide and Feulgen) provide evidence for DNA in or associated with the kinetosomes of S. tabogae or any other amitochondriate protist studied here.

By use of detergent-treated and spontaneously isolated mastigont systems of Snyderella, Stephanonympha and Caduceia, it was possible to show that DAPI stained the parabasal bodies, which are densely packed membranous Golgi complexes [2, 26]. The parabasal body apparently also binds or retains other DNA stains except for SYTOX. When the parabasal bodies were treated with detergent they did not stain with DAPI, nor did DAPI staining occur when the cells were pretreated with cold TCA.

The parabasal body is a permanent Golgi complex organized around a fiber that attaches it to the mastigont system in the Parabasalia (trichomonads and hypermastigids). The substances that are usually processed through these organelles have not been described for this class of protists, nor is the chemistry of this membranous-fibrous organelle system known. The same Golgi complex in calonymphids from Incisitermes snyderi does not bind DAPI or the other stains, nor do the numerous long, thin parabasal bodies in Trichonympha. This suggests that the staining is not due to an accumulation of the stains in the Golgi cisternae, but to binding to material in or on the cisternae. The stain concentration data (see Table 1) suggest a weak binding of DAPI to unidentified material in the parabasal body, analogous to its weak binding to microtubules. While detergent treatment dissolves part of the Golgi cisternae, some membrane is left. The absence of DAPI staining in these partially dissolved cisternae suggests that the basis for the staining is not the membrane itself, but is due to binding to variable quantities of unidentified material within the lumen of the cisternae. The inconsistent results across genera of calonymphids may be due to peculiarities of the microbial ecology of the Cryptotermes cavifrons gut versus

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**Table 1** Snyderella tabogae cell structure fluorescence with DAPI as a function of concentration*

<table>
<thead>
<tr>
<th>DAPI conc. (µM)</th>
<th>Structures stained</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.29</td>
<td>none</td>
</tr>
<tr>
<td>0.58</td>
<td>nuclei and epi- and endosymbiotic bacteria only</td>
</tr>
<tr>
<td>1.45</td>
<td>nuclei, bacteria and parabasal bodies</td>
</tr>
<tr>
<td>2.40</td>
<td>nuclei, bacteria, parabasal bodies, axostyles</td>
</tr>
</tbody>
</table>

*Cells were not treated with Triton X-100.
that of the two species of *Incisitermes*.

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**References**

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30. Sonneborn TM (1970) Determination, development and inheritance of RNA with the ciliary basal bodies of *Chlamydomonas reinhardtii*: evidence against a basal body location. Proc Natl Acad Sci USA 92:5129–5133