USE OF THE FLUOROCHROME 4′6-DIAMIDINO-2-PHENYLINDOLE IN GENETIC AND DEVELOPMENTAL STUDIES OF CHLOROPLAST DNA

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ABSTRACT

Use of the DNA-specific fluorochrome 4′6-diamidino-2-phenylindole (DAPI) makes it possible to examine in situ the structure of chloroplast DNA (chDNA) with the fluorescence microscope. This simplifies the study of genetic and developmental changes in chloroplast DNA. Three examples are presented. (a) Wild-type Euglena gracilis B contains several chloroplast DNA nucleoids per chloroplast. A yellow mutant lacking functional chloroplasts is similar, but such nucleoids are absent in an aplastidic mutant strain known from biochemical studies to have lost its chDNA. (b) In vegetative cells of the giant-celled marine algae Acetabularia and Batophora, only about a quarter of the chloroplasts have even one discernible chloroplast DNA particle, and such particles vary in size, showing a 30-fold variation in the amount of DNA-bound DAPI fluorescence detected per chloroplast. By contrast, 98% of chloroplasts in developing Acetabularia cysts contain chDNA, with as many as nine nucleoids per chloroplast. (c) DAPI-stained chloroplasts of chromophyte algae display the peripheral ring of DNA expected from electron microscope studies. However, these rings are not uniform in thickness, but are necklace-like, with the appearance of beads on a string. Since the multiple nucleoids in plastids of chromophyte algae also appear to be interconnected throughout the chloroplast, a common structural plan may underlie chDNA morphology in both groups of algae.

KEY WORDS Acetabularia · algae · chloroplast DNA · DAPI · Euglena

New techniques can often shed light on longstanding research questions. The recent availability of a new series of fluorescent compounds offers such an opportunity. The compounds in question are 33258 Hoechst (9, 13) and several derivatives of 4′6-diamidine-2-phenylindole (DAPI) (12). These compounds are highly specific in their binding to linear polymers with phosphate backbones, such as polyphosphates and DNA, and they exhibit a much enhanced fluorescence when bound to double-stranded DNA, particularly that with high AT content (10). Hence, aggregates of as little as $10^{-16}$ g of DNA, such as occur in yeast mitochondria, can easily be seen in situ with the fluorescence microscope (17).

DNA is also present in chloroplasts, but its study by light microscopy has been difficult. Only rarely has its presence been demonstrated unequivocally by Feulgen staining, and rather faint images were obtained using acridine dyes and fluorescence microscopy. However, after DAPI or Hoechst staining, chloroplast DNA (chDNA) is readily visible in the chloroplasts of such Volvocales algae as Chlamydomonas (4) and also in chloroplasts of higher plants (8). The purpose of the present account is to demonstrate some of the ways in which DAPI can be used as a probe to study chDNA morphology in both developmental and genetic investigations.

MATERIALS AND METHODS

The strains of algae employed for this study are listed in Table I. Euglena was maintained at 24°C in constant...
Sources of Algal Cultures

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetabularia calyculus</td>
<td>UTEX</td>
<td>(15)</td>
</tr>
<tr>
<td>Acetabularia crenulata</td>
<td>Lake Surprize, Fla.</td>
<td>(J. Zollner)</td>
</tr>
<tr>
<td>Batophora oerstedii</td>
<td>Lake Surprize, Fla.</td>
<td>(J. Zollner)</td>
</tr>
<tr>
<td>Ditylum brightwellii</td>
<td>Guillard (WHOI)</td>
<td>(7)</td>
</tr>
<tr>
<td>Codium fragile</td>
<td>Woods Hole</td>
<td></td>
</tr>
<tr>
<td>Euglena gracilis B</td>
<td>Schiff (Brandeis)</td>
<td>(11)</td>
</tr>
<tr>
<td>Y1BXD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W3BUL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sphacelaria sp.</td>
<td>UTEX LB 800</td>
<td>(15)</td>
</tr>
</tbody>
</table>

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were also extracted from the vegetative cell with a fine caps and squashed on slides. Chloroplasts of A. calyculus
were prepared in the same fashion, so that the chloro-
plasts could be studied within the vegetative cell. Simi-
larly, developing cysts were dissected from Acetabularia
caps and squashed on slides. Chloroplasts of A. calyculus
were also extracted from the vegetative cell with a fine
glass pipette, dispersed into a drop of Homogenizing
Medium on a slide, and processed by freezing and 3:1
fixation. Formalin-fixed material of Acetabularia crenu-
lata and Batophora oerstedii was used. Sphacelaria main-
tained in Erd-Schreiber Medium (15) at 24°C under
constant light and freshly collected Codium were fixed in
2% glutaraldehyde in seawater for 1 h at room tempera-
ture, then rinsed in distilled water and processed im-
mediately.

All slide preparations and other fixed materials were
subjected to ribonuclease digestion (bovine pancreatic
RNase, Sigma Chemical Co., St. Louis, Mo. 5X crystal-
line, 0.5 mg/ml in distilled water for 2 h at 37°C), a
procedure which had been found to enhance the clarity
of at least some types of DAPI-stained cell preparations.
Next, the material was rinsed in distilled water, stained in
0.5 μg/ml DAPI (Boehringer Mannheim Biochemi-
cals, Indianapolis, Ind.) in distilled water for 10 min at
room temperature, and then rinsed in several changes of
distilled water for 3 h at 4°C. The rinsed material was
mounted in Mcllvain's citrate-phosphate buffer, pH 5.5,
FIGURE 1  *Euglena gracilis* B, strain Y, BXD, a yellow mutant lacking functional chloroplasts but retaining chDNA, as seen by (a) transmitted light and (b) DAPI fluorescence. Black arrows indicate nuclei; white arrows indicate groups of chDNA nucleoids. Bar, 10 μm.

by Schiff and Epstein (11) and suggest the usefulness of DAPI.

**Acetabularia**

In a thorough examination of chloroplasts isolated from enucleated *Acetabularia* cells, Woodcock and Bogorad (18) documented an unexpected observation, i.e., only 20–35% of *Acetabularia* chloroplasts contained any detectable chDNA. The meaning of this observation and the question of how widespread this phenomenon might be among organisms whose cells have multiple chloroplasts are not clear.

Using DAPI to examine chDNA, we have confirmed their observations on cultured *Acetabularia* vegetative cells, and we have observed the same phenomenon in both *Acetabularia* and the related genus *Batophora* which were collected from the wild. 73% of the chloroplasts from vegetative cells lacked any detectable chDNA. 25% had only one DNA nucleoid per chloroplast, and 2% had two nucleoids. The size of such nucleoids varied considerably. Table II presents data from initial attempts to quantify this variation. The smallest particle observed had 2 U of fluorescence when stained with DAPI, while the largest had more than 60 U, a span of at least 30-fold. By comparison, the DAPI-stained nuclei of *Acetabularia* cysts had ~500 U of fluorescence. This value, however, can only be a very approximate indication of relative DNA content since the nuclei, being much larger, could not be measured accurately with this aperture combination, and, furthermore, the base composition of nuclear and chloroplast DNAs differs in *Acetabularia*.

The measurements in Table II were made on nearly mature vegetative stalks; in young germings there was also a tremendous range in nucleoid size, but a greater frequency of large nu-

FIGURE 2  *Euglena gracilis* B, strain W3RUL, a white mutant lacking chDNA as seen by (a) phase microscopy and (b) DAPI fluorescence. Black arrows indicate nuclei. Bar, 10 μm.
cleoids. One extreme example was an irregular V-shaped nucleoid which measured 1.5 μm in diameter when viewed end on, and 3.2 μm when rolled over to face view. Nevertheless, in both germling and older vegetative stalks, the proportion of chloroplasts containing nucleoids was similar to the 20–35% reported by Woodcock and Bogorad (18).

We also observed a radical change during cap formation. The proportion of Acetabularia chloroplasts having at least one DNA particle is much increased in the cytoplasm of developing cysts. At this stage in the life cycle, as many as 98% of the chloroplasts contain chDNA, with as many as nine particles per chloroplast (Fig. 3). This contrasts with a maximum of two particles per chloroplast in vegetative cells. Further studies are needed to determine how chDNA replication and distribution are altered at the transition from the vegetative to the reproductive state in this unique family.

To check whether DNA-free chloroplasts are characteristic of green algae with a siphonaceous growth stage, cells of Codium were also examined. No examples of chloroplasts lacking chDNA particles were found in an examination of several thousand chloroplasts in the utricles of this alga (Fig. 4). Thus, the chDNA deficiency in vegetative cells of Dasycladacean algae may be unique to that family, and its control relative to the life cycle presents an intriguing problem.

**TABLE II**

<table>
<thead>
<tr>
<th>Range of relative fluorescence units</th>
<th>No. of chloroplasts</th>
<th>Nucleoid size range</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2</td>
<td>238</td>
<td>*</td>
</tr>
<tr>
<td>2–10</td>
<td>26</td>
<td>&lt;0.2–0.2</td>
</tr>
<tr>
<td>11–20</td>
<td>19</td>
<td>—</td>
</tr>
<tr>
<td>21–30</td>
<td>16</td>
<td>—</td>
</tr>
<tr>
<td>31–40</td>
<td>9</td>
<td>—</td>
</tr>
<tr>
<td>41–50</td>
<td>11</td>
<td>0.7–0.9</td>
</tr>
<tr>
<td>51–60</td>
<td>6</td>
<td>—</td>
</tr>
<tr>
<td>&gt;60</td>
<td>1</td>
<td>—</td>
</tr>
</tbody>
</table>

Data are tabulated from four glycerol-mounted preparations of full-grown but still vegetative Acetabularia calyculus plants. Measurements were made on individual chloroplasts, either within the stalk or dissected from it (see Materials and Methods). The immediately adjacent unoccupied area was measured for each and used as a background correction.

* No chloroplast displaying <2 U of relative fluorescence had any detectable nucleoid. Fluorescence increased with increasing nucleoid size, as indicated by measurements made for two of the fluorescence categories.

**Chromophyte Algae**

chDNA has been demonstrated in a number of organisms by electron microscopy. The DNA usually appears as localized regions, "nucleoids," of tangled DNA fibrils lying between the photosynthetic lamellae. In the green algae, in *Euglena*, and in higher plants there is no known organization of the locations of these pockets, but in the Chromophyte algae (3), electron micrographs generally show the DNA pockets to lie near the ends of the ovoid chloroplasts. In at least two cases, it has been possible to demonstrate by serial sectioning of chloroplasts that the DNA lies in a ring just under the outermost chloroplast lamella (1, 6).

We have examined the chloroplasts of a number of Chromophyte algae using DAPI, and have found that the rings of DNA are easy to see both in situ and in isolated chloroplasts. DAPI staining...
Portion of Codium fragile utricle showing chloroplasts (small arrows), vacuole (v), and nucleus with one nucleolus (large arrow). Multiple DAPI-stained chDNA nucleoids are visible in each ellipsoidal chloroplast, as well as one largest starch grain. Photographed by (a) phase, (b) DAPI fluorescence, and (c) membrane autofluorescence, primarily, plus partial DAPI fluorescence. Bar, 5 μm.

revealed in addition that the rings are not bands of uniform diameter but have the appearance of closely spaced beads on a necklace (Fig. 5). The bead number varied with the ring size. Furthermore, the ring orientation clearly showed that the chloroplasts, at least in larger cells, had the plane of their rings parallel to the adjacent cell surface (Fig. 6).

The diameter of chDNA rings is not constant. This was apparent particularly in Sphaelaria, a filamentous brown alga where each branch tip is a growing point. In cells at or near the apex, DNA rings with hourglass shapes were frequently observed (Fig. 6), reminiscent of chloroplasts pinching in two as described by Bisalputra and Bisalputra (2). Assuming the smallest chloroplasts to be the most newly formed, we observed the chloroplasts and their DNA rings to be smallest in the apical cells, and increasingly larger as one progressed away from the apex. The minimal length of the ovoid ring in the smallest apical cell chloroplasts was 1.9 μm, while the length of the oval in the smallest chloroplast of a cell five cells removed from the apex (where cell division has ceased and the filament is four cells thick) was 3.1 μm. Farther back from the apex, the fully enlarged cells exhibited surfaces paved with larger, relatively uniform chDNA rings, each within its chloroplast.

Prospects

The value of DAPI as a stain for chDNA lies in its very high quantum efficiency of fluorescence, which is enhanced more than 10-fold by binding to DNA (10). It has very high specificity for DNA, which can be assisted by pretreating cells with RNase and PCA to remove interfering materials. There is increasing evidence that it binds most strongly and/or specifically to AT-rich DNA. This characteristic may account for its power in rendering chDNA visible since, where values are known, chDNA is higher in AT content than the nuclear DNA of the same cell. Thus, DAPI can be used as a tool for rapidly screening mutants, as with Euglena, or for assessing the presence and location of DNA, as with Acetabularia and Codium. It readily reveals the gross morphology of DNA in organelles, without having to resort to electron microscopy, as shown with the Chromophyte genera.

The nature of the stained material is easily demonstrated by applying DNase, which causes it to disappear. The autofluorescence of chloroplast membranes, even when chlorophyll has been extracted, confirms that the DAPI-stained structures lie within chloroplasts. One continuing question is whether one is also discerning mitochondrial DNA stained with DAPI when examining whole cells. That mitochondrial DNA can be seen in at least some organisms has been shown for yeast and trypanosomes. However, by criteria of morphology and cell location, we have observed no distinct identifiable mitochondrial DNA in the algae discussed here, with the possible exception of the diatom, Ditylum. Even the Euglena mutant lacking chDNA, which should be ideal for mitochondrial
DNA observation, has only a vague, finely divided glow when stained with DAPI. The explanation may lie in the fact that mitochondrial DNA genomes average 10-fold smaller than chDNA genomes. The mitochondrial DNA of yeast may be visible only because of its extremely high AT content (17).

The enumeration of DNA nucleoids per chloroplast is at best only a gross quantitation method. More precise quantitation presents another problem. Thanks to the affinity for AT-rich sequences, it is unlikely that DAPI can be used to quantitate differences in chDNA content between different organisms, since they vary in the base ratios of their chDNA and presumably also in the sizes of their AT-rich sequences. However, as the data in Table II suggest, it may well be possible to quantitate changes in chDNA within an organism, a problem on which we are currently working. With this methodology, one could observe directly the kinetics of chDNA increase with respect to the nuclear cell cycle and the effects on DNA morphology of various inhibitors known to block plastid DNA synthesis.

Previous examination of chDNA distribution in green algae belonging to the Volvocales has suggested that the plastid nucleoids are not discrete but rather irregularly shaped aggregates on what might be a continuous skein distributed throughout the large, cup-shaped chloroplast present in each cell (4). Euglena chDNA presents the same appearance, and a similar observation has been reported for chDNA of higher plants (8). If this proves to be true, the organization of chDNA in chlorophyte organisms might be more similar to that in chromophyte organisms than has previously been apparent. In both, localized condensations are only subunits of a more encompassing structure; in chromophytes, the beads on a string present a two-dimensional array, a ring around the plastid; in chlorophyte genera, the beads are more distant from each other and have a three-
FIGURE 6 UV fluorescence of ring-shaped DNA chromophores within chloroplasts of a Sphacelaria subapical cell. View is an optical section just beneath the cell wall. Bar, 5 μm.

dimensional array. Using DAPI, it should be possible to discover further examples of these patterns with ease, and this ultimately may lead to a better understanding of plastid DNA behavior.

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