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

ANNETTE W. COLEMAN. From the Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912

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 chlorophyte 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 long-standing 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 Volvocalean 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

TABLE I

Sources of Algal Cultures

Species	Source	Reference
Acetabularia calyculus	UTEX	(15)
Acetabularia crenulata	Lake Surprize, Fla. (J. Zollner)	
Batophora oerstedii	Lake Surprize, Fla. (J. Zollner)	
Ditylum brightwellii	Guillard (WHOI)	(7)
Codium fragile	Woods Hole	
Euglena gracilis B	Schiff (Brandeis)	(11)
Wild type		
Y_1BXD		
W_3BUL		
Sphacelaria sp.	UTEX LB 800	(15)

light of ~400 fc intensity in Volvox Medium (15) supplemented with 0.1% sodium acetate, 0.01% yeast extract, and 0.01% proteose peptone. To reduce the content of paramylum granules, which obscure chloroplast details, log cultures were transferred to Resting Medium (16). After 3 d, cells were harvested by centrifugation, squashed under a cover slip on slides in a drop of the Homogenizing Medium of Shephard (14) which was designed to preserve chloroplast structure in broken cells, and immediately frozen in liquid N_2. The cover slip was removed and the slide immersed in 3:1 ethanol:acetic acid. After fixation and dehydration through an alcohol series, slides were air dried.

Whole cells of *Ditylum* and *Acetabularia calyculus* were prepared in the same fashion, so that the chloroplasts could be studied within the vegetative cell. Similarly, 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 crenulata* and *Batophora oerstedii* was used. *Sphacelaria* maintained 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 temperature, then rinsed in distilled water and processed immediately.

All slide preparations and other fixed materials were subjected to ribonuclease digestion (bovine pancreatic RNase, Sigma Chemical Co., St. Louis, Mo. 5× crystalline, 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 Biochemicals, 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 McIlvain's citrate-phosphate buffer, pH 5.5, and examined immediately. Slides could be stored indefinitely in the cold after remounting in glycerol.

Where polyphosphate material was found, which fluoresces orange with DAPI rather than the blue-white characteristic of DAPI-DNA (4), a 1-h extraction in ice-cold 2% perchloric acid (PCA) to remove the interfering material was inserted after the RNase treatment. To insure that the DAPI-stained material in chloroplasts was DNA, slides which had already been examined were exposed to deoxyribonuclease (DNase I, DP OCA, Worthington Biochemical Corp., Freehold, N. J., 0.2 mg/ml in 10 mM Tris, pH 7.4, 1 mM $MgCl_2$ for 3 h at 37°C) and then restained and examined.

Observations were made on a Zeiss photomicroscope equipped with a 100-W mercury lamp providing epi-illumination for fluorescence microscopy. Zeiss filter combination 48 77 02 was used for observation of DAPI staining, and filter combination 48 77 06 for comparison of membrane autofluorescence. A Neofluor 100/1.30 oil objective was used for fluorescence measurements and for photography on Tri-X film. Fluorescence of DNA nucleoids in *Acetabularia* chloroplasts was measured with the same microscope modified for microspectrophotometry by addition of a 50-Hz light modulator and an MPM 01K photometer head attached to a PMI indicator. A lamp field stop of 12 μm diameter in the plane of the specimen and a measuring field diaphragm of 2 μm were used to measure relative fluorescence intensity in the transmission mode.

RESULTS AND DISCUSSION

Euglena

Euglena has been used extensively for the examination of chDNA characteristics, and stable chloroplast mutants are known. Biochemical studies have shown that some of these mutants, though lacking functional chloroplasts, retain chDNA, while others appear to have lost their chDNA complement entirely (5, 11). Since considerable labor is involved in the biochemical determination of DNA species, we examined both the wild type and several mutants of *Euglena* to see whether the picture obtained with DAPI staining would correspond with that presented by the biochemical data. This would provide a simpler alternative technique for screening mutants or following chDNA loss under bleaching conditions.

Wild-type *Euglena* contained numerous chDNA aggregates, clearly more than 20 per cell, and in one case at least 74. These flecks were often stringy in appearance rather than discrete. Likewise, a yellow mutant Y_1BXD (Fig. 1) contained numerous chDNA particles, but a white mutant W_3BUL (Fig. 2) contained no such particles. These observations agree with the biochemical results reported

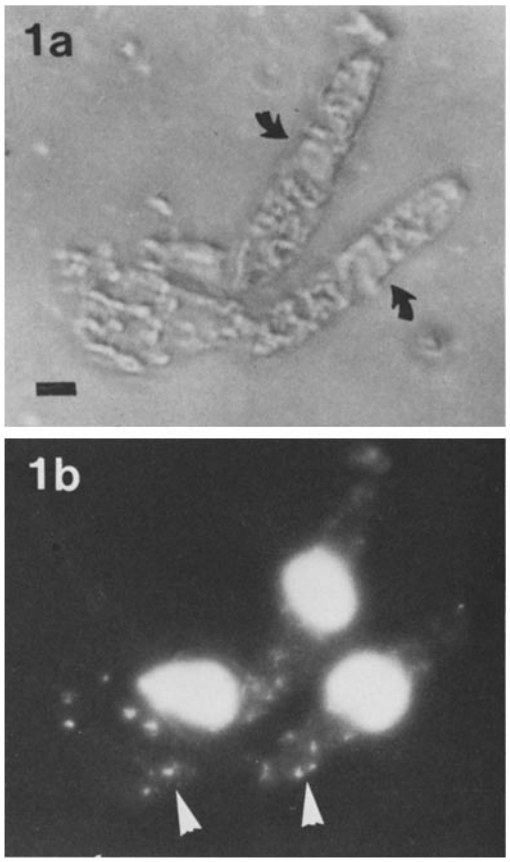

FIGURE 1 *Euglena gracilis* B, strain Y_1 BXD, a yellow mutant lacking functional chloroplasts but retaining chDNA, as seen by (*a*) transmitted light and (*b*) DAPI fluorescence. Black arrows indicate nuclei; white arrows indicate groups of chDNA nucleoids. Bar, 10 μm.

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 germlings there was also a tremendous range in nucleoid size, but a greater frequency of large nu-

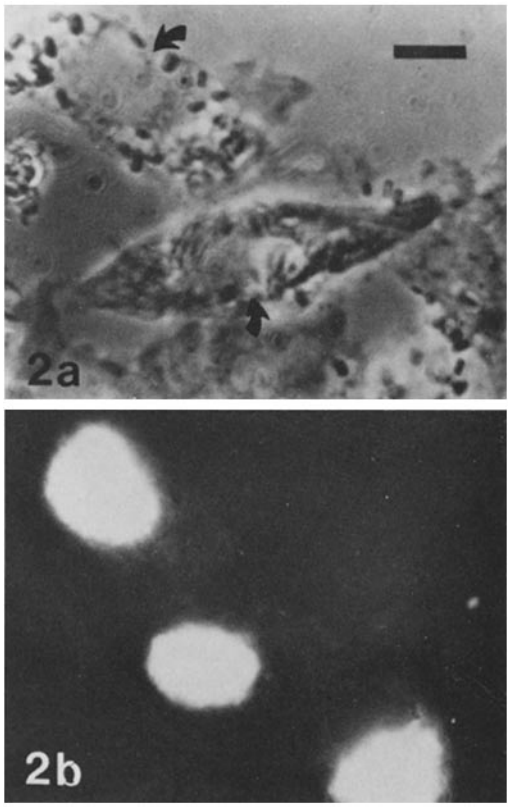

FIGURE 2 *Euglena gracilis* B, strain W_3BUL, a white mutant lacking chDNA as seen by (*a*) phase microscopy and (*b*) DAPI fluorescence. Black arrows indicate nuclei. Bar, 10 μm.

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

Fluorescence of DAPI-Stained Chloroplast DNA

Range of relative fluorescence units	No. of chloroplasts	Nucleoid size range
		μm
<2	238	*
2–10	26	<0.2–0.2
11–20	19	—
21–30	16	—
31–40	9	—
41–50	11	0.7–0.9
51–60	6	—
>60	1	—

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.

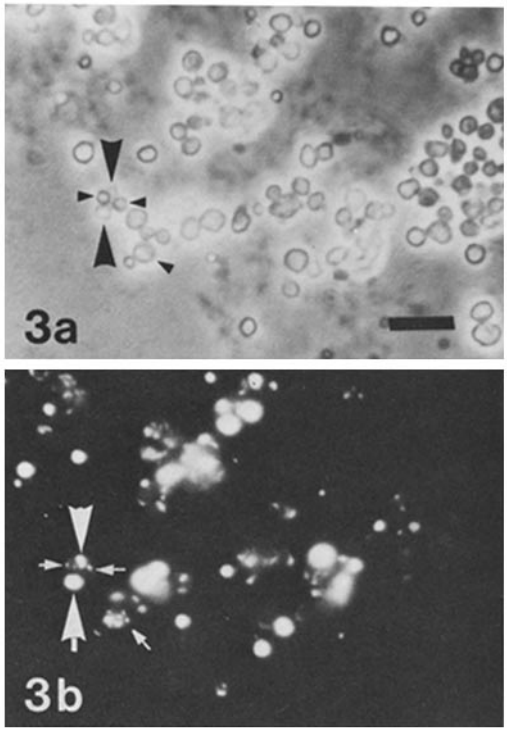

FIGURE 3 Portion of cytoplasm from multinucleate, developing cyst of *Acetabularia calyulus* squashed in Homogenizing Medium, fixed, and stained with DAPI. Secondary nuclei (large arrows) and chloroplasts containing multiple chDNA particles (small arrows) are visible. "Flaring" causes the nuclear diameters to vary with the plane of focus. Photographed by (*a*) phase microscopy and (*b*) DAPI fluorescence; only the more central area is illuminated with the UV beam. Bar, 20 μm.

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

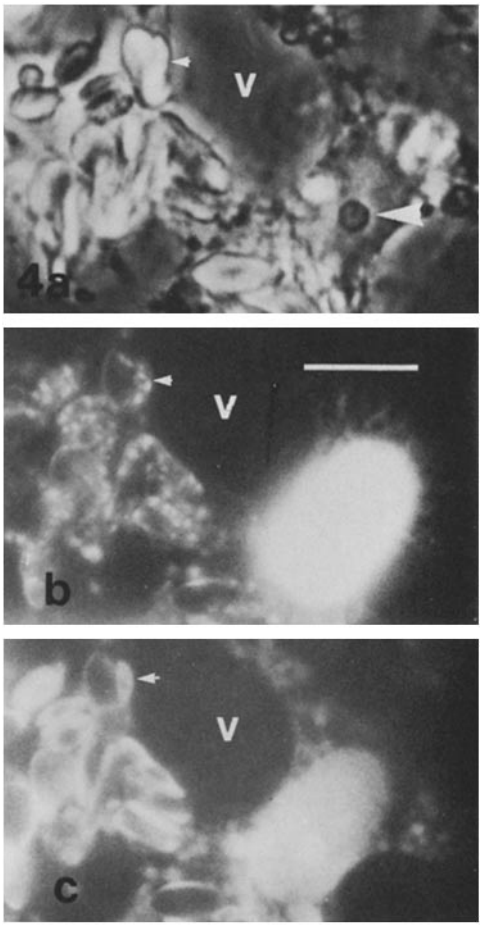

FIGURE 4 Portion of *Codium fragile* utricle showing chloroplasts (small arrows), vacuole (*v*), and nucleus with one nucleolus (large arrow). Multiple DAPI-stained chDNA nucleoids are visible in each ellipsoidal chloroplast, as well as one large starch grain. Photographed by (*a*) phase, (*b*) DAPI 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 *Sphacelaria*, 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

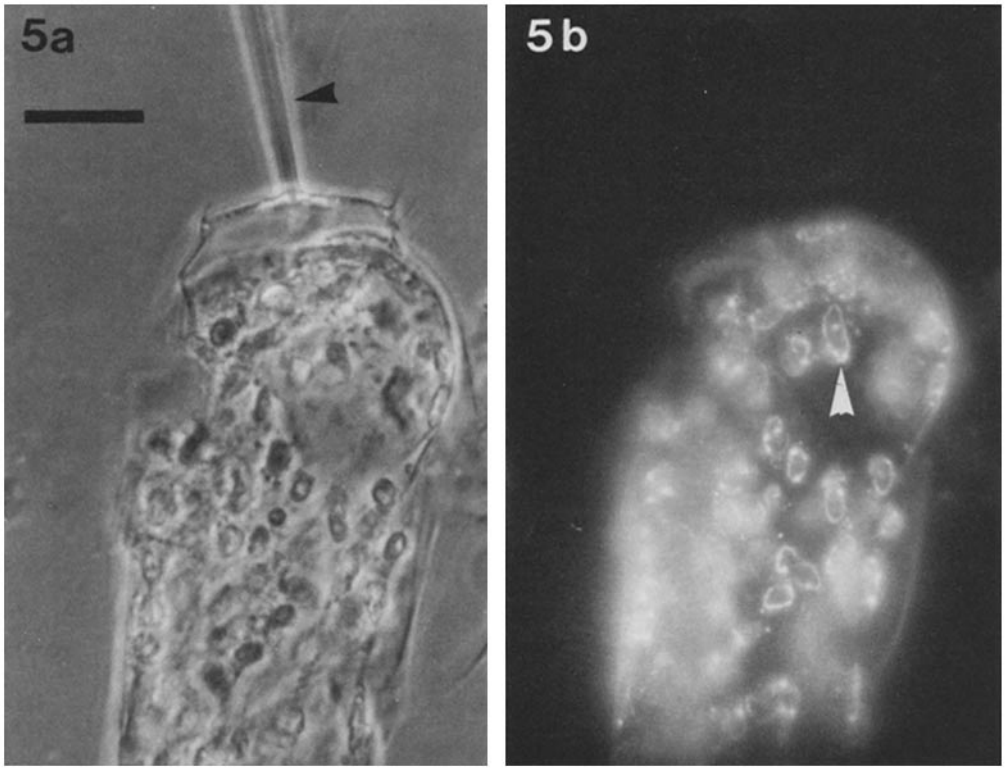

FIGURE 5 Portion of *Ditylum brightwellii* cell slightly crushed in Homogenizing Medium, fixed, and stained with DAPI. Note spine (black arrow) observed in phase (*a*) and irregular densities on chDNA rings (white arrow) visible with DAPI fluorescence (*b*). Isolated, single, bright particles in the cell may represent mitochondrial DNA. Bar, 10 μm.

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-

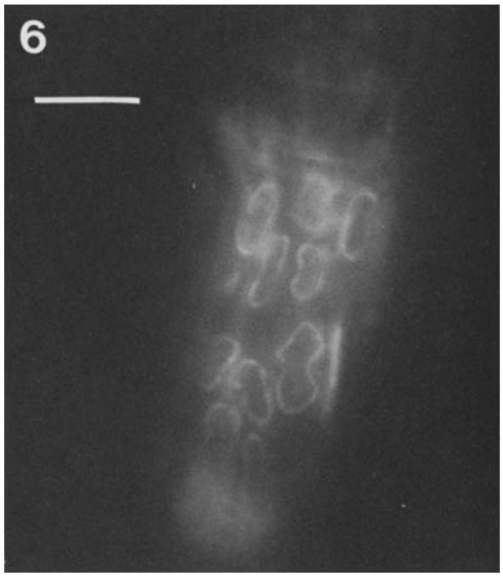

FIGURE 6 UV fluorescence of ring-shaped DNA chromophores within chloroplasts of a *Sphacelaria* subapical cell. View is an optical section just beneath the cell wall. Bar, 5 μm.

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.

The author gratefully acknowledges the gifts of algal material from Dr. R. R. L. Guillard, Dr. J. A. Schiff, and Ms. J. Zollner; the sample of DAPI from Dr. O. Dann; the technical assistance of M. Maguire; and the exploratory studies of Ms. C. Geffen.

This material is based upon work supported by the National Science Foundation under grants PCM 78-15783 and DEB-76-82919 as well as U. S. Public Health Service institutional grant 5-S07-RR 07085.

Received for publication 26 January 1979, and in revised form 4 April 1979.

REFERENCES

1. BISALPUTRA, T., and A. A. BISALPUTRA. 1969. The ultrastructure of chloroplast of a brown alga *Sphacelaria sp.* I. Plastid DNA configuration—the chloroplast genophore. *J. Ultrastruct. Res.* **29**:151–170.
2. BISALPUTRA, T., and A. A. BISALPUTRA. 1970. The ultrastructure of chloroplast of a brown alga *Sphacelaria sp.* III. The replication and segregation of chloroplast genophore. *J. Ultrastruct. Res.* **32**:417–429.
3. CHRISTENSEN, T. 1962. Alger. *In* Systematisk Botanik. Bind II. Nr. 2. T. W. Böcher, M. Lange, and T. Sorenson, editors. Munksgaard, Copenhagen. 178 pp.
4. COLEMAN, A. W. 1978. Visualization of chloroplast DNA with two fluorochromes. *Exp. Cell Res.* **114**:95–100.
5. EDELMAN, M., J. A. SCHIFF, and H. T. EPSTEIN. 1965. Studies of chloroplast development in *Euglena*. *J. Mol. Biol.* **11**:769–774.
6. GIBBS, S. P., D. CHENG, and T. SLANKIS. 1974. The chloroplast nucleoid in *Ochromonas danica*. I. Three-dimensional morphology in light- and dark-grown cells. *J. Cell Sci.* **16**:557–577.
7. GUILLARD, R. R. L. 1975. Culture of phytoplankton for feeding marine invertebrates. *In* Culture of Marine Invertebrate Animals. W. L. Smith and M. H. Chanley, editors. Plenum Publishing Corp., New York.
8. JAMES, T. W., and C. JOPE. 1978. Visualization by fluorescence of chloroplast DNA in higher plants by means of the DNA-specific probe 4′-6-diamidino-2-phenylindole. *J. Cell Biol.* **79**:623–630.
9. LATT, S. A. 1973. Microfluorometric detection of deoxyribonucleic acid replication in human metaphase chromosomes. *Proc. Natl. Acad. Sci. U. S. A.* **70**:3395–3399.
10. LIN, M. S., D. E. COMINGS, and O. S. ALFI. 1977. Optical studies of the interaction of 4′-6-diamidino-2-phenylindole with DNA and metaphase chromosomes. *Chromosoma (Berl.).* **60**:15–25.
11. SCHIFF, J. A., and H. T. EPSTEIN. 1968. The continuity of the chloroplast in *Euglena*. *In* The Biology of Euglena. D. E. Buetow, editor. Academic Press, Inc., New York.
12. SCHNEDL, W., A.-V. MIKELSAAR, M. BREITENBACH, and O. DANN. 1977. DIPI and DAPI: fluorescence banding with only negligible fading. *Hum. Genet.* **36**:167–172.
13. SETH, P. K., and A. GROPP. 1973. Study of constitutive heterochromatin with a new and simplified fluorescence staining technique. Genetica (*The Hague*). **44**:485–495.
14. SHEPHARD, D. C. 1970. Photosynthesis in chloroplasts isolated from *Acetabularia mediterranea*. *In* Biology of Acetabularia. J. Brachet and S. Bonotto, editors. Academic Press, Inc., New York.
15. STARR, R. C. 1978. The culture collection of algae at the University of Texas at Austin. *J. Phycol.* **14**(Suppl.):47–101.
16. STERN, A. I., J. A. SCHIFF, and H. T. EPSTEIN. 1964. Studies of chloroplast development in *Euglena*. V. Pigment biosynthesis, photosynthetic O_2 evolution and CO_2 fixation during chloroplast development. *Plant Physiol.* **39**:220–226.
17. WILLIAMSON, D., and D. FENNELL. 1975. The use of fluorescent DNA-binding agent for determination and separating yeast mitochondrial DNA. *In* Methods in Cell Biol. XII. Yeast Cells. D. Prescott, editor. Academic Press, Inc., New York.
18. WOODCOCK, C. L. F., and L. BOGORAD. 1970. Evidence for variation in the quantity of DNA among plastids of *Acetabularia*. *J. Cell Biol.* **44**: 361–375.