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Ultrastructural Localization of RNA in Cryptomonads

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Summary

In a previous study, DNA was localized in cells of two cryptomonads, *Pyrenomonas* sp. and *Cryptomonas ovata*, by use of immuno-gold technique. Of particular interest was the ultrastructural localization of DNA in the nucleomorph, supposed to be a vestigial nucleus of a former endosymbiont [HANSMANN P *et al.* (1986) Eur J Cell Biol 42: 152–160]. In the present paper, distribution of RNA in the same two organisms is reported. RNA was detected by the specific and very sensitive RNase-gold method. RNA could be demonstrated in all of the four plasmatic compartments of cryptomonad cells (cytoplasm, periplastidal compartment, mitochondrion, and plastid), although the amounts differed greatly in the respective compartments. In the nucleus, the condensed chromatin and the nucleolus were preferentially labeled. Intense labeling could also be found over the fibrillogranular region of the nucleomorph. This fact lends strong support to the supposition that the fibrillogranular body represents the structural and functional equivalent of a nucleolus and thus again supports the hypothesis that the nucleomorph represents a vestigial eukaryotic nucleus. In *Pyrenomonas* sp., gold-particle density over the nucleolus and the fibrillogranular body was quantitatively evaluated in order to compare their respective RNA synthesizing activities. Labeling density over the nucleolus was found to be 2.7 times higher and thus, on account of its greater volume, the nucleolus may contain 17 times more RNA than the fibrillogranular body of the nucleomorph.

Keywords: Cryptomonads; Nucleomorph; RNA localization; Enzyme-gold cytochemistry.

Abbreviations: BSA bovine serum albumin, ER endoplasmic reticulum, GA glutaraldehyde, SSC standard saline citrate, SSCB SSC containing BSA.

1. Introduction

Cryptomonads are a small group of unicellular eukaryotic algae with no known direct relatives (CORLISS 1987). Electron-microscopical studies have revealed that cryptomonad cells are composed of four different plasmatic compartments (cytoplasm, periplastidal compartment, plastid, and mitochondrion), separated from each other by two membranes in every case. Cryptomonads are unique in possessing a periplastidal compartment (*sensu* GIBBS) that is totally separated from the cytoplasm by a so-called periplastidal ER (GIBBS 1962, 1979, 1981 a, GREENWOOD *et al.* 1977). This compartment contains starch grains, some vesicles and membrane tubules, 80 S-type ribosomes, and a body limited by a double-membrane with a few pores. On account of fine structural similarity of this body to that of a nucleus, it was termed "nucleomorph" (GREENWOOD 1974, GREENWOOD *et al.* 1977). In a nonstructured matrix the nucleomorph comprises electron dense particles of unknown composition and function together with a fibrillo-granular body resembling a nucleolus (GREENWOOD *et al.* 1977, GILLOTT and GIBBS 1980, SANTORE 1982). These unusual structural features caused several authors to suggest that cryptomonads might have arisen from a cytosymbiotic system between a heterotrophic zooflagellate and a phototrophic *eukaryotic* alga, the latter being the cytosymbiont (*e.g.*, GIBBS 1981 b, WHATLEY 1981). (This hypothesis may also apply to all other algal groups possessing plastids that are surrounded by more than two membranes, *i.e.*, *Euglenophyta, Dinophyta,* and *Chromophyta.*) The demonstration that the nucleomorph indeed represent a vestigial eukaryotic nucleus would provide conclusive evidence in support of this hypothesis. Recently (HANSMANN *et al.* 1985, LUDWIG and GIBBS 1985), DNA could be detected in the nucleomorph, and furthermore, it could be shown that the DNA distribution is

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similar to the DNA topology in the nucleus (HANSMANN *et al.* 1986). Previous attempts to demonstrate RNA or DNA in the fibrillogranular body of the nucleomorph using BERNHARD's (1969) regressive stain led to ambigious results (GILLOTT and GIBBS 1980, SANTORE 1982). The aim of the present study was to check the presence of RNA in the fibrillogranular body by means of BENDAYAN's (1981) RNase-gold method. A preliminary account of the results presented here has been given as part of a published lecture (HANSMANN *et al.* 1987).

2. Material and Methods

2.1. Culture Methods

Pyrenomonas sp. [*P.* sp.; probably *Pyrenomonas salina* Santore; Basionym: *Cryptomonas salina* Wislouch (*cf.*, SANTORE 1984); the same organism has been used in our previous studies (HANSMANN *et al.* 1985, 1986)] was a gift of Dr. P. EMSCHERMANN, University of Freiburg, Federal Republic of Germany, and was cultured in half strength "f" medium (GUILLARD 1975). *Cryptomonas ovata* (*C. ovata*) was obtained from the Sammlung von Algenkulturen Göttingen, Federal Republic of Germany, and was grown on desmidiacean medium (SCHLÖSSER 1982). Both cultures were maintained at 20 °C with a 12-hour dark/12-hour light cycle at a light intensity of 3,000–4,000 lux. For fixation, cells were harvested 14 days after inoculation (log-phase), 2 hours after beginning of a light cycle.

2.2. Processing of Cells for Electron Microscopy

For conventional electron microscopy, the cells were GA/OsO_4 fixed, ethanol dehydrated, and Epon embedded as described previously (HANSMANN *et al.* 1986). Micrographs were taken with a Zeiss EM 10 CR electron microscope.
For cytochemical localization, *C. ovata* was prefixed in the culture medium by adding GA and cacodylate buffer, pH 7.2, to final concentrations of 0.5% and 10 mM, respectively, at room temperature. After 30 minutes the cells were collected by centrifugation at 500 g for 5 minutes, further fixed in 4% formaldehyde (freshly prepared from paraformaldehyde), 0.5% GA, 50 mM phosphate buffer, pH 7.4, for 2.5 hours, and washed 5 times in phosphate buffer. *P.* sp. was collected without prefixing, resuspended in the same fixation medium as *C. ovata* but containing 0.5 M sucrose, fixed for 3 hours, and washed 5 times in a decreasing sucrose series in phosphate buffer. The fixed cells of both species were preembedded in 1.5% low-gelling-temperature agarose (SeaPlaque, FMC Corporation, U.S.A.) at 37 °C, dehydrated in a graded ethanol series at 4 °C, and finally embedded in Epon 812 (GLAUERT 1974). Sections (Ultracut microtome, Reichert-Jung, Federal Republic of Germany) were mounted on carbon-reinforced Formvar-coated 200 mesh hexagonal nickel grids.

2.3. Preparation of Protein-Gold Complexes

Colloidal gold was prepared by reduction of tetrachloroauric acid with citrate according to the method of FRENS (1973). This procedure resulted in gold particles of 12–16 nm in diameter.
RNase-gold complexes were prepared as described by BENDAYAN (1981). 10 ml gold suspension, adjusted to pH 9.0 with 0.1 M K_2CO_3, were rapidly added to 0.1 ml aqueous RNase solution (containing 0.5 mg RNase A, type XII-A, Sigma Chemical Company, U.S.A.) in a siliconized glas tube and gently mixed for 2 minutes. The RNase-gold complexes were centrifuged at 56,000 g for 30 minutes at 4 °C. The loose pellet was resuspended in 3 ml SSCB (standard saline citrate: 10 mM sodium citrate, pH 7.4, 140 mM NaCl; containing 0.1% bovine serum albumin, Boehringer Mannheim, Federal Republic of Germany) and stored at 4 °C.
BSA-gold was prepared in the same way but using BSA instead of RNase, and adjusting the gold sol to pH 7.3.

2.4. Cytochemical Labeling

Sections were labeled using the following protocol (all steps at room temperature): 5 minutes bidistilled water (b.w.); 1 hour 0.2 M sodium metaperiodate; two times 5 minutes SSC; 30 minutes RNase-gold complex in SSCB (50 µl droplets); three times 5 minutes SSC; three times 5 minutes b.w.; air drying. Staining of the sections: 5 minutes b.w.; 7 minutes 2% aqueous uranyl acetate; three times 1 minute b.w.; 1 minute lead citrate; three times 1 minute b.w.; air drying.
Several controls for labeling specificity were carried out: Incubation with BSA-gold; addition of RNA (1 mg/ml) to the RNase-gold complex; preincubation of the sections with RNase A (1 mg/ml in SSC) for two times 1.5 hours at 35 °C.

2.5. Quantitative Evaluation

The density of labeling after incubation with RNAse-gold was evaluated according to BENDAYAN (1981). In the case of *P.* sp., the number of gold particles present over the nucleolus and the fibrillogranular region of the nucleomorph was counted and the corresponding surface area was measured according to the principles of WEIBEL (1969). A total of 10 acceptable micrographs for each compartment were evaluated at a final magnification of 27,500. The labeling density was calculated by division of the number of gold particles by surface area (μm^2). Values for the relative volumes of the nucleolus and the fibrillogranular body were taken from work of BALTES (1987).

3. Results

Combined fixation with 4% formaldehyde and 0.5% glutaraldehyde (without osmium postfixation) resulted in a satisfactory ultrastructural preservation of the cells. Fixation with either fixative, when employed alone, led to insufficient results. Pretreatment of the sections with sodium metaperiodate improved the specificity of the labeling by lowering background staining. The specificity of the RNase-gold complexes was checked by control experiments (not shown). The labeling was greatly diminished by preincubation with RNase as well as by addition of RNA to the enzyme-gold complex. With BSA-gold only very few randomly scattered gold particles could be observed.
For both organisms studied (*P.* sp. and *C. ovata*), the overall labeling was similar. A high density of gold particles was found over the cytoplasm (Figs. 1 *a* and 3 *a*). The labeling was particularly concentrated along

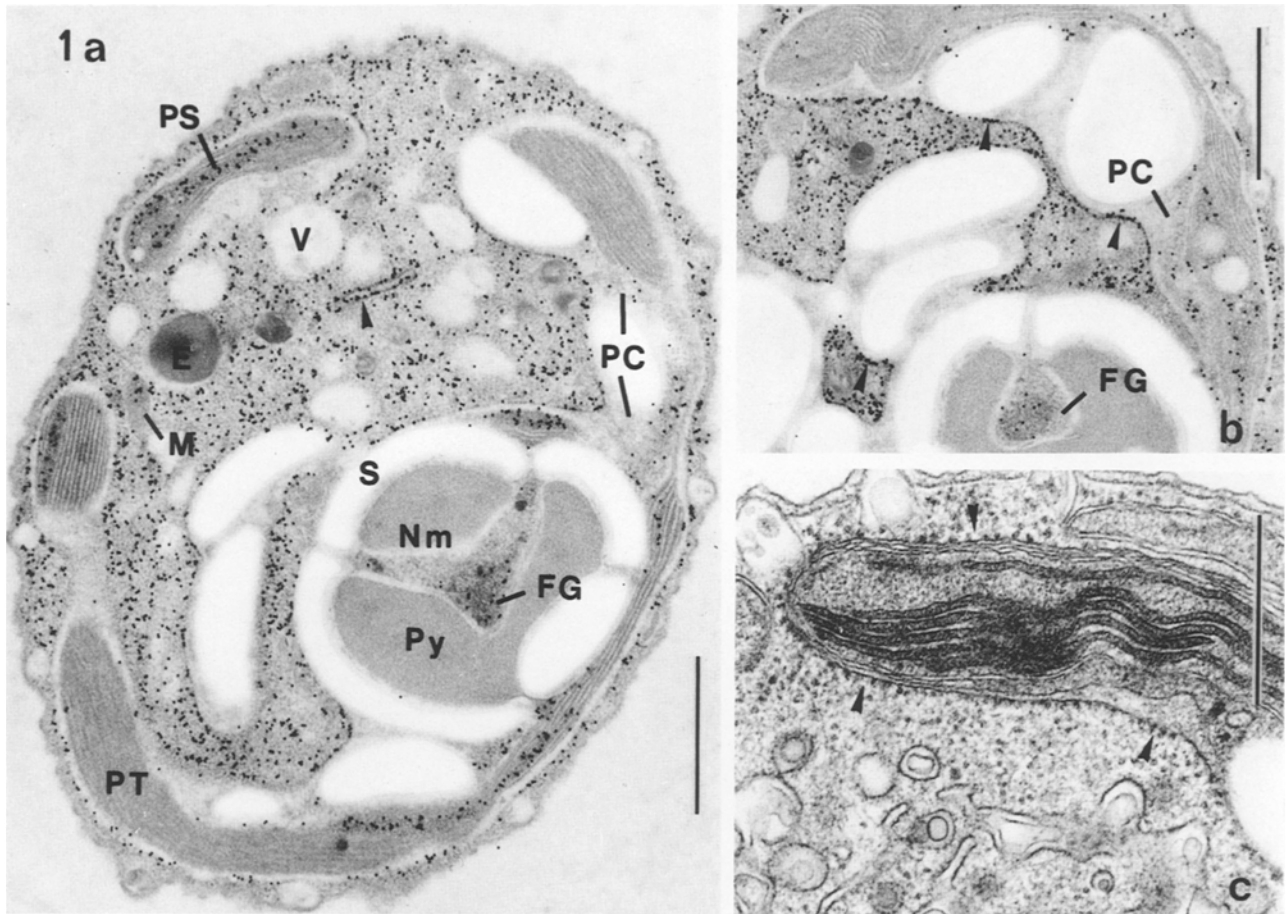

Fig. 1. Localization of RNA on Epon sections of *Pyrenomonas* sp. (*a*, *b*). *a* Overview of a whole cell. Cytoplasm, rER (arrowhead), the fibrillogranular body (*FG*), and the plastid stroma (*PS*) are intensely labeled. The plasm of the periplastidal compartment (*PC*) shows less intense labeling. Only few gold particles are present over the mitochondrion (*M*) and almost no label occurs over vacuoles (*V*), ejectisomes (*E*), starch grains (*S*), thylakoids (*PT*), and the pyrenoid (*Py*). *b* The outer membrane of the periplastidal ER shows intense labeling (arrowheads), whereas the inner membrane is almost devoid of gold particles. *c P.* sp. fixed with GA and OsO_4. The cytoplasmic side of the outer periplastidal ER membrane is studded with ribosomes (arrowheads). Bars 1 µm (*a*, *b*), 0.5 µm (*c*)

the outer membrane of the periplastidal reticulum (rough endoplasmic reticulum bordering the periplastidal compartment) (Fig. 1 *b*), which in GA/OsO_4-fixed cells appears to be studded with ribosomes on the cytoplasmic side (Fig. 1 *c*). Vacuoles and ejectisomes were not labeled. In *P.* sp., the cytoplasm of the periplastidal compartment showed only faint labeling (Fig. 1 *a* and *b*), whereas in *C. ovata* it was more intensely labeled (Fig. 3 *a*). Starch grains were almost devoid of gold particles. A high density of gold particles could be observed over the plastid stroma but the thylakoids and the pyrenoid were only very faintly labeled. Only few gold particles were present over the mitochondrion (Figs. 1 *a* and 3 *a*).

In the nucleus, the gold particles were preferentially located over the nucleolus. The condensed chromatin was less intensely labeled and labeling over the dispersed chromatin was very weak (Figs. 2 *a* and 3 *a*). In the nucleomorph, gold particles were mainly located over the fibrillogranular body, the matrix and dense particles being almost completely devoid of label (Figs. 2 *b*, *c* and 3 *b*–*d*). In *P.* sp., the nucleomorph lies in a groove of the pyrenoid and is almost entirely surrounded by the plastid envelope (the pyrenoid is part of the plastid). There is only a small zone where the nucleomorph is directly associated with the cytoplasm of the periplastidal compartment and where the nucleomorph envelope is perforated by pores (Fig. 2 *d*). In some cases this area of the nucleomorph was also labeled (Fig. 2 *c*).

In *P.* sp., the density of labeling over the nucleolus and the fibrillogranular body of the nucleomorph was eval-

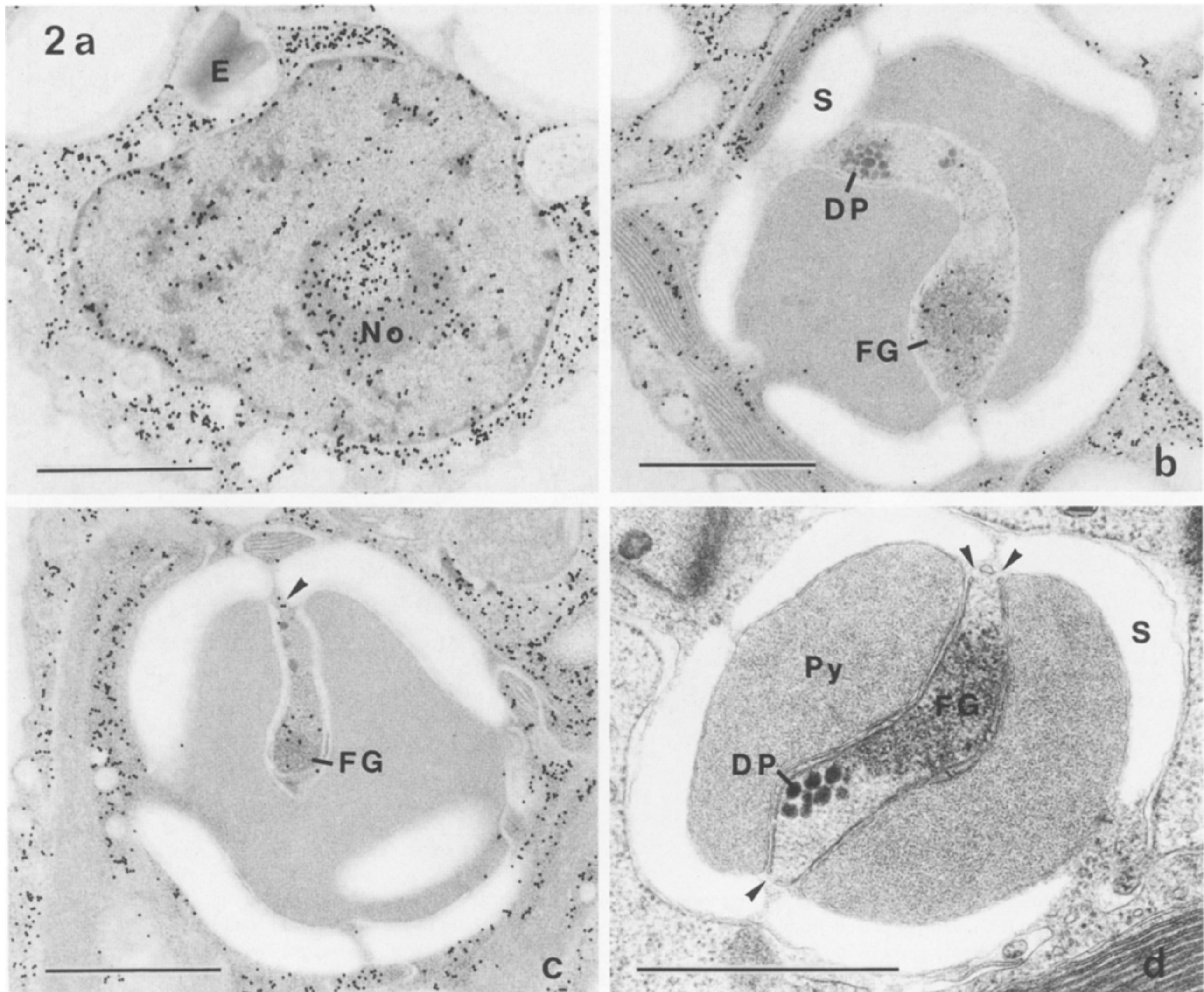

Fig. 2. Demonstration of RNA in the nucleus and the nucleomorph of *P.* sp. (*a–c*). *a* Nucleus showing intense labeling over nucleolus (*No*) and condensed chromatin. The dispersed chromatin appears to be only faintly labeled. *b* In the nucleomorph, gold particles are particularly concentrated over the fibrillogranular body (*FG*). Dense particles (*DP*) and matrix are almost devoid of label. *c* Few gold particles are present over the contact zone between the nucleomorph and the cytoplasm of the periplastidal compartment (arrowhead). *d P.* sp. after GA/OsO_4-fixation. The nucleomorph lying in a groove of the pyrenoid (*Py*), only contains pores in a narrow region facing the cytoplasm of the periplastidal compartment (arrowheads). Bars 1 μm

uated quantitatively. Gold particle density over the nucleolus was 75 per μm^2, and over the fibrillogranular body 28 per μm^2. Both values far exceeded the background labeling over starch grains, ejectisomes, and vacuoles (aproximately 2 particles/μm^2).

4. Discussion

The RNase-gold method has been shown to be suitable for ultrastructural localization of RNAs in both animal and plant cells. This cytochemical method was found to be very sensitive and, under appropriate conditions, highly specific (*e.g.*, BENDAYAN 1981, BENDAYAN and PUVION 1984, PICHE *et al.* 1984, CHAREST *et al.* 1985, CHENICLET and CARDE 1987). In the cryptomonad cells investigated here (*Pyrenomonas* sp. and *Cryptomonas ovata*), the high specificity was confirmed by several control experiments, and this was further supported by the fact that all organelles known to contain RNA were labeled (ribosomes, nucleolus, chromatin), whereas RNA-free structures were almost devoid of gold particles (starch grains, vacuoles, ejectisomes).

4.1. Ribosomes

The cryptomonads are unique among the algal groups in possessing a periplastidal compartment, which sup-

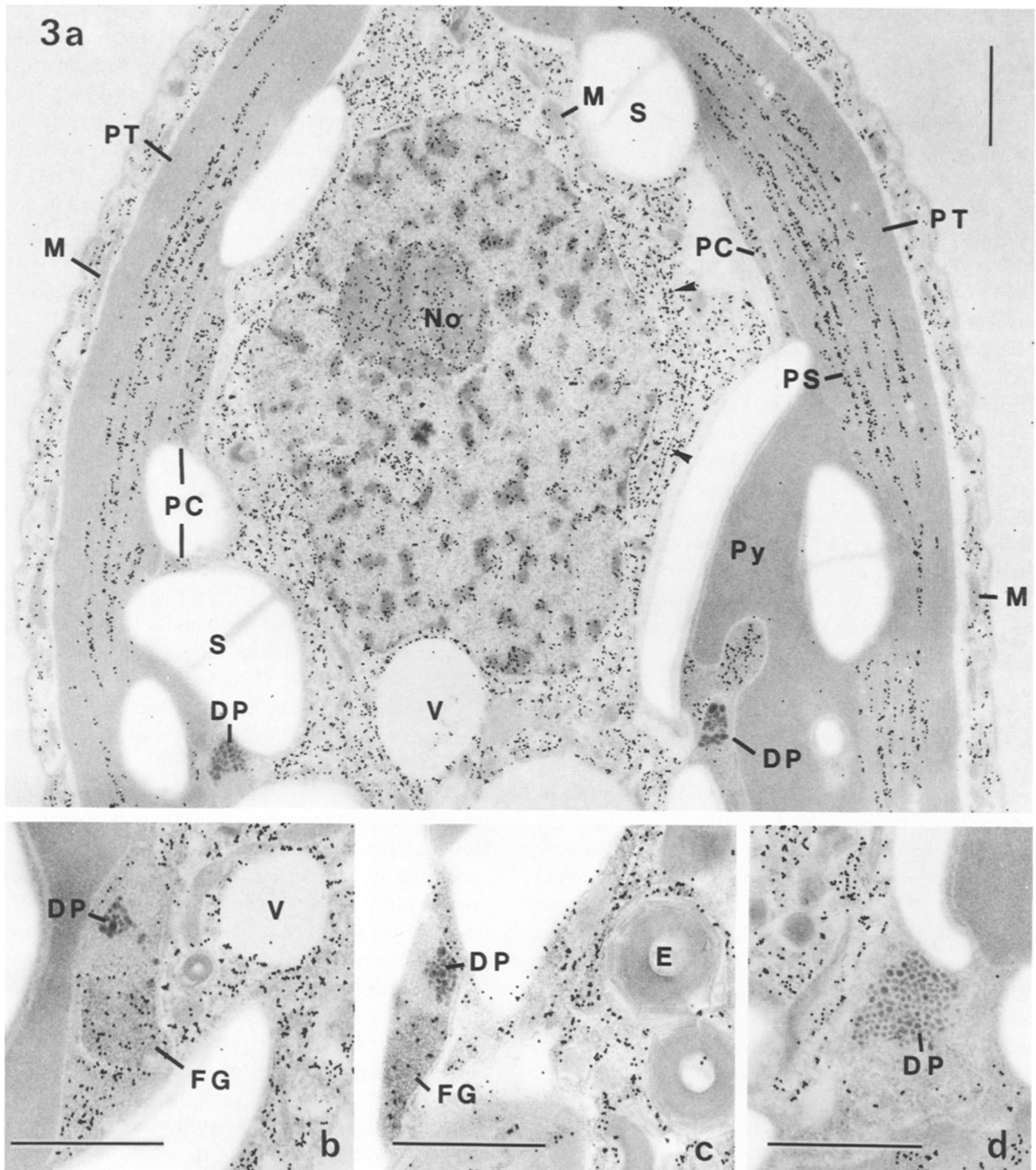

Fig. 3. Epon sections of *Cryptomonas ovata* incubated with RNase-gold. *a* Overview of a cell. Cytoplasm, rER (arrowheads), nucleolus (*No*), condensed chromatin, plastid stroma (*PS*), and parts of the periplastidal compartment (*PC*) are intensely labeled. Only a few gold particles are present over the mitochondrion (*M*). Vacuoles (*V*), starch grains (*S*), dense particles of the nucleomorph (*DP*), the thylakoids (*PT*), and the pyrenoid (*Py*) are almost without label. *b–d* In the nucleomorph, the labeling is preferentially localized over the fibrillogranular region (*FG*). Over the nucleomorph matrix, only very few gold particles are seen. Ejectisomes (*E*) and nucleomorph dense particles (*DP*) are almost devoid of label. Bars 1 μm

posedly represents the remains of the cytoplasm of a symbiotic eukaryotic alga (GIBBS 1981 b, WHATLEY 1981). Besides starch grains, tubules, vesicles, and the nucleomorph (see below), the periplastidal compartment contains ribosomes which are of the same size as those found in the cytoplasm (22 nm) and are thus probably of the eukaryotic type (GIBBS 1962, SEPSENWOL 1973, GILLOTT and GIBBS 1980). Quantitative evaluation of ribosomal numbers in *P.* sp. revealed that, in the cytoplasm, the ribosomal density is 4 times higher than in the periplastidal compartment (relative surface area of starch grains substracted). Furthermore, on account of the relative volumes, the whole cell has 37 times more ribosomes in the cytoplasm than in the periplastidal compartment (BALTES 1987). Since the RNase-gold method in the cytoplasm preferentially stains ribosomes (BENDAYAN 1981), the difference in labeling density over the two plasmatic compartments observed in *P.* sp. (this study) is in good agreement with the difference in ribosomal density (BALTES 1987). In particular, the outer membrane of the periplastidal ER was found to be highly labeled, indicating a high density of ribosomes, which could also be observed by conventional electron microscopy. In contrast, the inner membrane is devoid of gold particles. This uncommon distribution of ribosomes over the periplastidal ER has also been reported in previous publications and led to the conjecture that the total plastid-periplastidal compartment complex lies *in* a rough ER cisterna consisting of the outer membrane of the periplastidal ER, and that the inner membrane represents the plasma membrane of the former cytosymbiont (WHATLEY 1981). It has also been suggested that the ribosomes present on the outer periplastidal ER membrane are responsible for the synthesis of nuclear-coded proteins which are destined for the periplastidal compartment and/or the plastid (GIBBS 1979). A prerequisite for this would be that genetic information had been transferred from the plastid or the periplastidal compartment to the nucleus.

4.2. Nuclei and Nucleoli

The nucleus is well known as the main cellular compartment of RNA synthesis. Correspondingly, it invariantly contains considerable amounts of RNA. Within the nucleus, the nucleolus is responsible for transcription of rDNA, processing of pre-rRNAs, and formation of preribosomes. Therefore, highest concentrations of RNA are generally found in the nucleolus (BENDAYAN 1981). Cryptomonad cells are thought to possess two nuclei, the cell nucleus and a degenerate nucleus termed the nucleomorph (GREENWOOD 1974, GREENWOOD *et al.* 1977). It has been suggested that the fibrillogranular body lying in the nucleomorph may represent the structural and functional analogue of the nucleolus (GREENWOOD *et al.* 1977, GILLOTT and GIBBS 1980, SANTORE 1982). Small areas containing DNA presumably coding for rRNA could be found in both the nucleolus and the fibrillogranular body (HANSMANN *et al.* 1986). GILLOTT and GIBBS (1980) and SANTORE (1982) have attempted to demonstrate the presence of RNA in the fibrillogranular body using BERNHARD's (1969) regressive staining technique. However, as the technique applied has been shown to be not entirely specific (MOYNE 1980), and as the results reported were to some extent contradictory, final proof appeared desirable. In the present paper, RNA could be demonstrated unequivocally in the fibrillogranular body of the nucleomorph by means of the RNase-gold method. The dense particles, which are also embodied in the nucleomorph, have been assumed to be of viral nature containing RNA (GILLOTT and GIBBS 1980) or to represent condensed chromatin (MORRALL and GREENWOOD 1982). Recent reports showing them to contain neither DNA (HANSMANN *et al.* 1986) nor RNA (this study) mean however that the real nature of these nucleomorph inclusions must be reconsidered.

In *P.* sp., the elongated nucleomorph lies in a deep invagination of the pyrenoid leaving only a small contact zone between the cytoplasm of the periplastidal compartment and the nucleomorph. It is this contact area to which the nucleomorph pores are confined. A few gold particles could be observed in this area indicating the presence of some RNA, presumably associated with preribosomes. These may be synthesized and assembled in the fibrillogranular body, situated in the centre of the nucleomorph, and have to be transported through the pores of the nucleomorph envelope.

Since the main aim of this study was to reveal the composition and function of the nucleomorph fibrillogranular body, a quantitative evaluation of the labeling density over this compartment as compared to that over the nucleolus was carried out for *P.* sp. Particle density measured over the nucleolus was 2.7 times higher than that for the fibrillogranular body. The nucleolus had previously been shown to be 6.1 times larger than the fibrillogranular body (BALTES 1987, HANSMANN *et al.* 1987). Thus, altogether there may be about 17 times more RNA in the nucleolus than in the fi-

brillogranular body, which might suggest that the former compartment has a much higher capacity to produce ribosomes. This result agrees well with the volume ratios of cytoplasm and the periplastidal compartment, and with the ratio of ribosome numbers in these two compartments (see above, BALTES 1987).

4.3. Nature of the Nucleomorph

In order to explain the origin of complex plastids (plastids surrounded by more than 2 membranes in *Euglenophyta, Dinophyta, Chromophyta,* and *Cryptophyta*), the serial endosymbiotic hypothesis has been modified so as to suggest that the plastids of these algal groups have arisen from eukaryotic cytosymbionts rather than prokaryotic ones (GIBBS 1981 b, WHATLEY 1981). An important piece of evidence in favour of this hypothesis would be the unequivocal establishment of the cryptomonad nucleomorph as a vestigial eukaryotic nucleus. There are several main criteria for defining a nucleus: mitotic division, double membrane envelope containing pores, presence of DNA and RNA. Furthermore, nuclei actively synthesizing ribosomes contain a nucleolus. The cryptomonad nucleomorph now appears to meet all these requirements. Division, although in an unusual primitive mode, could be demonstrated (MCKERRACHER and GIBBS 1982, MORRALL and GREENWOOD 1982). Furthermore, the nucleomorph is limited by a double membrane with pores (GREENWOOD *et al.* 1977, GILLOTT and GIBBS 1980). DNA is present (HANSMANN *et al.* 1985, LUDWIG and GIBBS 1985) and displays a topology similar to DNA in the nucleus (HANSMANN *et al.* 1986). Finally, in the present study, RNA could be demonstrated especially in the fibrillogranular body, which again supports the conjecture that this structure represents the nucleomorph nucleolus (GREENWOOD *et al.* 1977, GILLOTT and GIBBS 1980, SANTORE 1982) being responsible for synthesis of the ribosomes localized in the periplastidal compartment. In summary, these facts all support the hypothesis that the cryptomonad cell has arisen by a secondary cytosymbiotic event involving two eukaryotes and thus is composed of two eukaryotic compartments, namely, the *host cytoplasm* containing the nucleus and the mitochondrion as well as the *cytosymbiont cytoplasm* harboring the vestigial nucleus (the nucleomorph) and the plastid.

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