NUCLEAR AND ORGANELLE RNA SYNTHESIS IN *OCHROMONAS:* THE EFFECTS OF LIGHT

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INTRODUCTION

In a companion study on the site of synthesis of chloroplast and mitochondrial RNA (7), it was shown that when dividing greening cells of *Ochromonas danica* were fed a radioactive RNA precursor, both the chloroplast and mitochondria as well as the nucleus were heavily labeled after a short labeling time, whereas a lag was observed in the labeling of the cytoplasmic matrix. These data were interpreted to mean that organelle RNA is synthesized within the organelles themselves, whereas the RNA of the cytoplasm is synthesized in the nucleus and subsequently transferred to the cytoplasm. However, since it is possible to interpret such radioautographic data in other ways, the present experiment was designed to answer more directly the question of where chloroplast RNA is synthesized. Preliminary studies had shown that when cells of *Ochromonas* are starved in a substrate-free medium, they are able to develop a small green chloroplast when placed in the light, even though they are incapable of further cell divisions. Under such conditions, it seemed likely that the only kind of RNA in the cell which would show a light-induced increase in its rate of synthesis would be chloroplast RNA. If this is so and if all chloroplast RNA is synthesized *in situ,* then one would expect that the only cell structure to show a light-induced increase in RNA synthesis would be the chloroplast. Consequently, in this study, dark-starved and light-induced nondividing cells of *Ochromonas* were labeled with tritiated orotic acid for a short interval and the amount of labeled RNA in each cell structure was determined by electron microscopic radioautography. For comparison purposes, similar observations were made on dark-grown and light-induced dividing cells. Contrary to expectations, it was observed that even in nondividing cells, light stimulates RNA synthesis in the nucleolus as well as in the chloroplast. However, other results obtained add further support to the hypothesis that chloroplast and mitochondrial RNA are synthesized *in situ.*

MATERIALS AND METHODS

Culture and Radioautographic Methods

Stocks of *Ochromonas danica* Pringsheim (17) were obtained from the Culture Collection of Algae and Protozoa at Cambridge. All cultures were grown at 26°C either under fluorescent lamps at an intensity of 450 ft-c. or in light-tight boxes. Cell counts and chlorophyll analyses were made as described previously (5). For the experiments on dividing cells, cultures were grown in the dark on Aaronson and Baker's (1) complete *Ochromonas* medium. Chloroplast development was induced by placing a culture of dark-grown logarithmically dividing cells in the light. To obtain cultures of nondividing cells, 10 ml of a dark-grown culture containing 6×10^6 cells per ml were added to 90 ml of substrate-free *Ochromonas* medium (1) and left in the dark. This transfer was

done in dim green light (Kodak Wratten safelight filter, series 7). Under these conditions, cell division ceases after 2–3 days. Chloroplast development in the absence of cell division was induced by placing such a dark-starved culture in the light 3 days after adding the cells to the substrate-free medium.

Four cultures were used in the radioautographic experiments: (*a*) a culture of dark-grown dividing cells which contained 5.1×10^6 cells per ml and 0.4×10^{-10} mg chlorophyll *a* per cell, (*b*) a culture of dark-grown dividing cells (a duplicate of culture *a*) which had been illuminated for 19 hr prior to the experiment and contained 4.9×10^6 cells per ml and 5.4×10^{-10} mg chlorophyll *a* per cell, (*c*) a culture of dark-starved, nondividing cells which contained 3.8×10^6 cells per ml and 1.1×10^{-10} mg chlorophyll *a* per cell, and (*d*) a culture of dark-starved nondividing cells (a duplicate of culture *c*) which had been preilluminated for 12 hr and contained 3.7×10^6 cells per ml and 5.0×10^{-10} mg chlorophyll *a* per cell. Cells from each culture were collected by gentle centrifugation and resuspended in either complete or substrate-free medium containing 50 μCi per ml of orotic acid-5-^{3}H (isotope from the Radiochemical Centre, Amersham; specific activity, 4.6 Ci/mmole) The two light-induced cultures were allowed to incorporate isotope in the light for 30 min before being fixed for electron microscopy. The dark-grown and dark-starved cultures were similarly exposed to isotope for 30 min but in complete darkness. It should be noted that all manipulations of the dark-grown cultures prior to fixation were done either in darkness or in dim green light. Aliquots from each culture were fixed for 2 hr in cold 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.1, rinsed thoroughly in cold buffer, and postfixed for 2 hr in cold 2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.3. The cells were then embedded in small agar blocks, dehydrated in an ethanol series, followed by epoxy-propane, and embedded in Araldite. For radioautography, uniform silver sections were cut and picked up on Formvar-coated nickel grids. The sections were coated with recently manufactured Ilford L-4 emulsion by the loop method of Caro and van Tubergen (4) and exposed at 2°C in the presence of a drying agent for 10 months. Development of the emulsion was done in D-19 for 5 min at 20°C. The grids were examined unstained in a Philips EM 200 electron microscope operated at 80 kv.

Ribonuclease digestion controls were performed by two different methods. After labeling, aliquots of the two dividing cultures were fixed in 1.6% glutaraldehyde and embedded in glycol methacrylate as described previously (7). Thin sections of this material were digested in ribonuclease A (Sigma Chemical Co., St. Louis, Mo., crystallized five times) with and without a cold 5% trichloroacetic acid (TCA) rinse, and in buffered distilled water with and without a cold TCA rinse following the schedule outlined in the previous paper (7). Aliquots of the nondividing cultures were fixed in 10% formalin in 0.1 M phosphate buffer, pH 7.3, for 40 min on ice. The fixed cells were rinsed thoroughly in cold buffer and suspended in ribonuclease A (1 mg/ml, pH 6.7) or in buffered distilled water at 38°C for a 2 hr digestion period. Both digested and untreated cells were postfixed in 2% osmium tetroxide for 2 hr and prepared for radioautography as described above.

Quantitation of Results

Ochromonas danica is a small unicellular flagellate averaging 11.4 μ long and 8.4 μ wide, exclusive of the flagella and tail. In these experiments the level of labeling was low, the number of grains per individual cell section ranging from 0 to 10. In order to make quantitative comparisons among the four cultures, it was necessary to record every cell section, the unlabeled as well as the labeled ones, on each grid analyzed. To do this, the number and distribution of grains in every cell section in four different grids were recorded for each of the four cultures, giving a total of 5960 cell sections analyzed. On each grid, it was necessary to photograph every cell section (usually 50–60) in the first section of the ribbon to serve as part of the sample of cells needed to determine the relative volume occupied by each cell organelle and the mean area per cell section. Subsequently, much of the recording of the presence or absence of grains and their location in each cell section was done by eye. However, all cell sections containing mitochondrial, cytoplasmic, or chloroplast grains were photographed, and the visually recorded location of the grains was confirmed on the printed micrographs. In determining the location of a grain, an arbitrary rule was made that if over half of a grain fell within the boundary of an organelle, the grain originated in that organelle.

To determine the relative volume occupied by each cell organelle in each type of a cell, 200–250 cell sections from each culture were photographed as described above at an initial magnification of 2300 and the micrographs were printed on 8 × 10 inch paper at a final magnification of 18,400. These micrographs were traced in their entirety on high-quality tracing paper and each organelle was cut out, and the organelles were collected in groups, and weighed. Since the cell sections traced are random sections through different cells of a population, this method gives a good estimate of

the per cent of the cell volume occupied by each cell organelle.

The total area occupied by each organelle in the cells analyzed from each culture equals the mean area per cell section *times* the number of cell sections analyzed *times* the per cent volume of the organelle. Mean area per cell section was determined from the same micrographs used for the relative volume determinations by the tracing and weighing method, and was found to be approximately the same for each of the four cell cultures (30.7 μ^2 ± 5%). This average value was used in all calculations. The number of background grains per unit area was also determined directly from the same representative micrographs of each culture.

An independent check was made to determine whether the volumes of the cells grown under the four different experimental conditions were indeed similar. Living cells were photographed in a Zeiss phase-contrast microscope at an initial magnification of 870 and printed at a magnification of 3500. Appropriate measurements were made on the prints on 80–100 cells from each culture, and the cell volume was approximated by representing the cell as a prolate spheroid plus a small cone (the tail base). This method confirmed that the cells grown under different culture conditions differed only slightly in mean cell volume (Table III).

RESULTS AND DISCUSSION

Ribonuclease digestion studies were performed on thin sections of glutaraldehyde-fixed glycol methacrylate-embedded cells or on whole formalin-fixed cells prior to embedding. In both cases, digestion with ribonuclease reduced the number of cell grains to background level, whereas the cells or sections extracted with warm distilled water alone showed no reduction in the number of cell grains. Rinsing the water-extracted sections with cold 5% TCA also did not lower grain counts, indicating that all unincorporated precursors had been removed from the cells during the fixation and embedding procedures. It is concluded that in each of the four cultures studied, the observed radioactivity is in RNA.

Table I shows the effect of light on RNA synthesis in dividing cells of *Ochromonas*. Both dark-grown and light-induced cells were labeled with tritiated orotic acid for 30 min and the concentration of labeled RNA in each cell compartment was determined. A look at the results for the dark-grown cells will show that the nucleolus is heavily labeled and that both the nucleoplasm and mitochondria are moderately well labeled. The small proplastid and the cytoplasmic matrix are only lightly labeled (about double the background level). A look at the light-induced cells will show that light causes an increase in the concentration of labeled RNA in all cell compartments. Chloroplast labeling is increased more than fivefold, nucleolar and nucleoplasmic labeling is double that of the dark-grown cells, and mitochondrial labeling is increased slightly. Since the volumes of the nucleolus, nucleoplasm, and mitochondria are approximately the same in the dark-grown and the light-induced cells (Table III), changes in the concentration of labelled RNA in each structure directly reflect changes in the total amount of labeled RNA in each structure. The chloroplast, however, increases 3.4-fold in volume in the first 19 hr in the light (Table III). Thus the total amount of chloroplast RNA synthesized during the 30 min labeling period is approximately 20 times greater in the light-induced cells than in the dark-grown cells.

TABLE I

*Specific Activity (grains/unit area) of Different Cell Compartments, after Labeling with Orotic Acid-5-^{3}H for 30 min. Dividing Cells.**

Cell compartment	Dark cells	19-hr light cells	Ratio‡ light/dark
	grains/1000 μ^2		
Nucleolus	173	403	2.4
Nucleoplasm	54	95	1.8
Chloroplast	8	26	5.5
Mitochondria	30	39	1.3
Remaining cytoplasm	8	12	2.0
Leucosin vacuole	3	6	—
Background	4	4	—

* Total grain count for dark cells, 419 grains; for 19-hr light cells, 458 grains.

‡ Before determining ratios, absolute grain counts were corrected for background grains.

Table II shows the effect of light on RNA synthesis in nondividing cells of *Ochromonas*. Dark-starved cells do incorporate orotic acid into RNA, but at a considerably lower rate than do the dark dividing cells. The concentration of grains over the the nucleoplasm is two-fifths that found in the dividing cells, whereas nucleolar labeling is reduced to one-fifth that observed in dark dividing cells.

This greater reduction of nucleolar RNA synthesis in nondividing cells is in line with the preferential inhibition of ribosomal RNA synthesis commonly observed in step-down cultures of both prokaryotic and eukaryotic cells (8, 12, 14, 16). In these dark nondividing cells, there is no detectable synthesis of chloroplast RNA, nor is the level of labeling of the cytoplasmic matrix above background. As far as the author is aware, this is the first time that it has been possible to demonstrate by the use of radioautographic techniques and a short labeling time that labeled RNA is present in the cell's mitochondria before any labeled RNA from the nucleus can be detected in the cytoplasm. This is excellent evidence that mitochondrial RNA is synthesized *in situ*, a hypothesis already well supported by a variety of studies (15, 18, 24, 26–29, 32, 33).

When dark-starved, nondividing cells of *Ochromonas* are placed in the light, chlorophyll synthesis commences after a short lag period and continues for 24–36 hr before ceasing. In this interval, the cell synthesizes a small chloroplast of normal ultrastructure. *Ochromonas* is an ineffectual photosynthesizer and, under the conditions of growth employed, is incapable of growing autotrophically, so that chloroplast development takes place in the absence of any cell division. It was expected that in this starved system, the chloroplast would be the only cell organelle to show a light-induced increase in RNA synthesis. However, it can be seen in Table II that light caused an increase in nucleolar as well as in chloroplast labeling. Nucleoplasmic labeling stayed constant, whereas mitochondrial labeling declined. Possibly chloroplast RNA synthesis proceeded at the expense of mitochondrial RNA synthesis. In these cells also all extranuclear grains are localized over either the chloroplast or the mitochondria.

TABLE II

*Specific Activity (grains/unit area) of Different Cell Compartments after Labeling with Orotic Acid-5-^3H for 30 min. Nondividing Cells.**

Cell compartment	Dark cells	12-hr light cells	Ratio‡ light/dark
	grains/1000 μ^2		
Nucleolus	37	60	1.8
Nucleoplasm	25	25	1.0
Chloroplast	4	13	∞
Mitochondria	17	12	0.7
Remaining cytoplasm	6	4	—
Leucosin vacuole	5	3	—
Background	5	3	—

* Total grain count for dark cells, 495 grains; for 12-hr light cells, 441 grains.

‡ Before determining ratios, absolute grain counts were corrected for background grains.

Because there is an increase in nucleolar as well as in chloroplast labeling in the light-induced, nondividing cells, this study did not provide evidence for the *in situ* synthesis of chloroplast RNA in the manner anticipated. However, two other observations strongly support the hypothesis that chloroplast RNA is synthesized *in situ*. The first is the fact, already noted, that when light-induced nondividing cells are exposed to orotic acid-^3H for a short time, the chloroplast and mitochondria are significantly labeled whereas the cytoplasmic matrix is not. This is good evidence that chloroplast RNA is synthesized *in situ* and not transferred from the nucleus. Secondly, an analysis of the distance of the chloroplast grains from the peripheral chloroplast nucleoid (for details of method, see 6) shows that in both the light-induced, dividing cells and the light-induced, nondividing cells, the labeled chloroplast RNA is concentrated near the chloroplast nucleoid, approximately half of the grains being localized directly

TABLE III

Volume of Cell Occupied by Different Cell Compartments

Culture conditions	Mean cell volume	Nucleolus	Nucleoplasm	Chloroplast	Mitochondria	Lipid granules	Remaining cytoplasm	Digestive vacuoles	Leucosin vacuole
	μ^3					%			
Dark, dividing	422	0.6	2.9	2.2	5.8	0	31	4.8	53
Light, dividing	398	0.6	2.8	8.0	6.8	0	36	3.3	42
Dark, nondividing	437	0.7	3.6	3.0	10.9	3.3	47	15	16
Light, nondividing	445	0.7	3.6	7.8	9.1	2.9	46	12	17

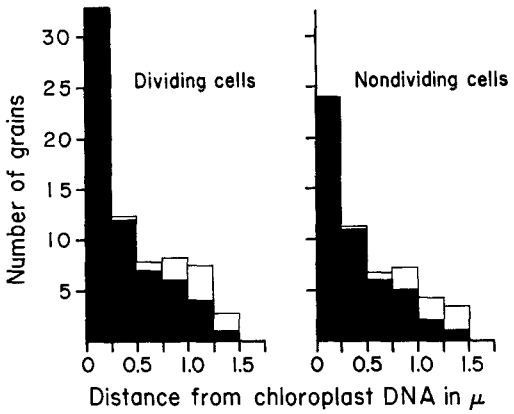

FIGURE 1 Distance of the labeled chloroplast RNA from the chloroplast DNA after labeling light-induced cells with orotic acid-5-^{3}H for 30 min. The solid black rectangles are the actual number of grains observed, and the white rectangles represent the additional number of grains which would have been observed at each distance if all chloroplast sections had been at least 3.5 μ long.

over the nucleoid (Fig. 1). It has been demonstrated previously by electron microscopic radioautography that all the chloroplast DNA is located in this peripheral ring-shaped nucleoid (20). Thus it is concluded that in both the dividing and nondividing cells the labeled chloroplast RNA is synthesized within the chloroplast using the chloroplast DNA as template.

It is of interest to consider what kinds of chloroplast RNA are synthesized within the developing chloroplast and whether there might be a difference between dividing and nondividing cells. It is now well established that chloroplast ribosomal RNA is synthesized in the chloroplast coded by chloroplast DNA (19, 23), and there is also some evidence that chloroplast transfer RNA and messenger RNA are synthesized within the chloroplast as well (2, 22, 25). In the previous study on greening dividing cells of *Ochromonas* (6, 7), it was postulated that the labeled RNA which was observed in the chloroplast concentrated at the chloroplast nucleoid after a 30 min labeling period was probably largely chloroplast ribosomal RNA because of the large increase in the number of chloroplast ribosomes which occurs during chloroplast development. It seemed likely that, during chloroplast development in starved, nondividing cells, there might not be an increase in the number of chloroplast ribosomes and that the labeled chloroplast RNA observed in these cells might be entirely transfer and messenger RNA. However, counts of the number of chloroplast ribosomes per unit area showed that, even in the nondividing cells, there was a threefold increase in the total number of ribosomes per chloroplast during the first 12 hr in the light. Similar counts on the dividing cells showed that there was a fivefold increase in the number of ribosomes per chloroplast during the first 19 hr in the light. Thus in nondividing as well as dividing cells, chloroplast ribosomal RNA may form a large fraction of the RNA synthesized in the chloroplast during chloroplast development.

The main observation of the present study, namely that in *Ochromonas*, light, in addition to markedly stimulating RNA synthesis in the chloroplast, also increases the rate of RNA synthesis in the nucleolus in both nondividing and dividing cells and in the nucleoplasm in dividing cells, is in good general agreement with the results of biochemical studies on other plant systems. Ingle (11) has studied RNA synthesis in developing radish cotyledons and has shown that light stimulates the synthesis of both cytoplasmic ribosomal RNA and chloroplast ribosomal RNA, although there is a fourfold greater stimulation of chloroplast RNA synthesis. Similarly, Bogorad (3) has observed that, in dark-grown maize leaves exposed to light for 3 hr, there is a small increase in the specific activity of the cytoplasmic ribosomes (1.5-fold) as well as a more marked increase in the specific activity of the chloroplast RNA as compared with the dark controls. Zeldin and Schiff (30, 31) have studied the effect of light on RNA synthesis in nondividing cells of *Euglena* and have shown that in the light-induced cells there is a greater incorporation of P^{32} into both nonchloroplastic (mainly ribosomal) RNA and chloroplast RNA than there is in the dark-grown cells. Recently Hoober and Blobel (9) have shown that cells of the *y-1* mutant of *Chlamydomonas reinhardi*, which have been exposed to light for 6 hr, have increased numbers of both cytoplasmic and chloroplast ribosomes when compared with dark-grown cells. Thus there is now evidence, in a variety of plant cells, that light increases the rate of RNA synthesis in both the nucleus and the chloroplast. In fact, the search for a system where chloroplast RNA would be the only type of RNA synthesized in the cell has to date proved futile (13).

Recent studies employing specific inhibitors of protein synthesis on 70S and 80S ribosomes indicate that, although some chloroplast proteins

are synthesized in the chloroplast on chloroplast ribosomes, other proteins are synthesized on cytoplasmic ribosomes and subsequently transferred to the chloroplast (10, 21). It seems likely that the light-induced increase in nucleolar and nucleoplasmic RNA synthesis observed in this study is related to the demands of the chloroplast for an increased synthesis on cytoplasmic ribosomes of proteins needed either directly or indirectly for its development.

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