IN VIVO SPECIFIC LABELING OF *CHLAMYDOMONAS* CHLOROPLAST DNA

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ABSTRACT

When *Chlamydomonas reinhardi* is supplied with (methyl-^{3}H)-thymidine, radioactivity is incorporated specifically into chloroplast DNA Chromatographic analysis of the products of enzymatic hydrolysis of the DNA reveals that only thymidine monophosphate has been labeled. Use of thymidine-6-^{3}H yields an identical result. If thymidine-^{3}H monophosphate is supplied, a small amount of radioactivity is incorporated into both nuclear and chloroplast DNA in proportion to the abundance of these DNA components. These observations are consistent with earlier suggestions that algae lack cytoplasmic thymidine kinase, but that the enzyme is present within their chloroplasts.

INTRODUCTION

The use of radioactively labeled bases and nucleosides has provided one of the most useful tools for the study of nucleic acid metabolism in many types of cells Unfortunately, attempts to coerce intact algae to incorporate exogenous pyrimidines into DNA in sufficient quantity to examine DNA metabolism have been unsuccessful (Ray and Hanawalt, 1964; Ray, 1964; Chiang, 1968, Davies, 1969). In the case of *Euglena*, labeling with uracil, uridine, or deoxyuridine results in moderate incorporation of radioactivity into RNA but little incorporation into DNA (Ray, 1964). Several investigators have shown in a variety of algal species that small amounts of labeled thymidine can be incorporated into chloroplasts but not into nuclei (Steffensen and Sheridan, 1965, Chapman et al , 1966; Stocking and Gifford, 1959). This was demonstrated by administering tritiated thymidine to cell cultures preparing autoradiograms, and showing that tritium decays were closely associated with the chloroplasts, but that very few could be detected in association with nuclei Steffensen and Sheridan (1965) were able to show that the incorporated radioactivity was sensitive to DNase and resistant to RNase, but Chapman et al (1966) found that 25% of the incorporated radioactivity was also RNase sensitive. Although these experiments indicate that some tritiated thymidine is incorporated into chloroplast DNA, they have not yet provided quantitative information on the metabolic fate of this precursor

The green alga *Chlamydomonas* has chloroplast DNA which can be separated from nuclear DNA on the basis of guanine-cytosine content in isopycnic cesium chloride gradients (Ris and Plaut, 1962, Sager and Ishida, 1963). Using this basic technique, we have examined the incorporation of tritiated thymidine into the DNA of *Chlamydomonas reinhardi*.

MATERIALS AND METHODS

Strains

Chlamydomonas reinhardi (90-) was obtained from K.-S. Chiang, and UVS 1-7 from D. R. Davies. Descriptions of the UVS strains are given by Davies (1967).

Growth Conditions

Cells were inoculated into 3/10 HSM (Sueoka et al, 1967) to a concentration of $4\,5 \times 10^4$ cells/ml. Radioactive precursors were added in all experiments at the time of inoculation. The cultures were grown on a rotary shaker at 22°C and illuminated continuously by cool white fluorescent lamps; the intensity at the top of the shaker was adjusted to 1000 ± 200 ft-c. A mixture of 95% air-5% carbon dioxide was bubbled through each culture. Cultures were harvested when they attained densities of $2\text{–}4 \times 10^6$ cells/ml as determined by a Coulter counter.

Lysate Preparation and Isopycnography

Cells were harvested by centrifugation at 3000 *g*, washed twice in NET buffer (0.1 M NaCl, 0.001 M ethylenediaminetetraacetate (EDTA), 0 01 M Tris, pH 7.6), and resuspended in 2 ml of NET in cellulose nitrate centrifuge tubes Sarkosyl NL 90 was added to a final concentration of 2%. The sample was heated to 45°C for 15 min with occasional shaking Cesium chloride and additional NET buffer were added to give a volume of 8 ml with a mean refractive index between 1 3995 and 1.4025 (At the lower refractive index, *Chlamydomonas* chloroplast DNA forms a peak in the center of the isopycnic gradient; at the upper value, chloroplast DNA is displaced toward the top of the gradient, and nuclear DNA bands near the center.) The tubes were centrifuged at 37,000 rpm for 36 hr at 25°C in a Beckman type-40 rotor (Beckman Instruments, Inc, Fullerton, Calif.). After centrifugation, fractions were collected from each tube, and the radioactivity in each fraction was determined by adding 12 ml of cold trichloroacetic acid, and collecting the precipitate on 2 4 cm Whatman GF/C glass fiber filters. These were washed twice with distilled water, dried, 4 ml of scintillation fluid were added, and the radioactive content of each was determined in a Packard liquid scintillation spectrometer.

Thin Layer Chromatography

100 μg of calf-thymus DNA was added to the pooled DNA-containing fractions from preparative CsCl equilibrium gradients, the mixture was brought to 0 3 M KOH and incubated overnight at room temperature to hydrolyze RNA. DNA was precipitated with cold 5% trichloroacetic acid and collected on Millipore HA filters (Millipore Corporation, Bedford, Mass). The washed filters were treated with DNase I. (The reaction mixture contained in 0.2 ml:0.8 μmoles Tris, 0 4 μmoles $MgCl_2$, 70 μg of the enzyme, at pH 7.9.) After 4 hr of incubation at 37°C, venom phosphodiesterase I (0.01 ml of 4 mg/ml in 1 mM NaCl and 4 mg/ml bovine serum albumin) was added and incubation was continued for 2.5 hr. The sample was evaporated to dryness, taken up in 0.1 ml of methanol, and chromatographed on PEI-cellulose as described by Randerath and Randerath, 1965. After drying the plates, nucleotide spots were located with a Mineralite, cut out, and radioactivity was determined as above in a Packard liquid scintillation spectrometer.

Materials

Sarkosyl was a gift of Geigy Chemical Corp. (Ardsley, N Y), and methotrexate a gift of Lederle Laboratories (American Cyanamid Co., Lederle Laboratories Div., Pearl River, N. Y). Other materials were purchased as follows: DNase (B grade), Calbiochem, San Diego, Calif; thymidine-6-^{3}H (9.7 Ci/mmole), (methyl-^{3}H)-dTMP (10 1 Ci/mmole), and (methyl-^{3}H)-dTTP (10.35 Ci/mmole), Schwarz/Mann, Orangeburg, N Y, (methyl-^{3}H)-thymidine (15 Ci/mmole) and adenine-^{3}H (6 mCi/mmole), New England Nuclear Corp, Boston, Mass., Phosphodiesterase I (*C. adamantus*), Worthington Biochemical Corp., Freehold, N. J; PEI plates, Brinkman Instruments Inc., Westbury, N. Y., Mineralite, Ultra-Violet Products, Inc., San Gabriel, Calif

RESULTS

When *Chlamydomonas* whole-cell DNA is banded in an equilibrium CsCl density gradient in the analytical ultracentrifuge, three distinct components (α, $\rho = 1.723$; β, $\rho = 1.695$, γ, $\rho = 1\ 715$) are resolved (Chiang and Sueoka, 1967) The α band, containing 85–90% of the DNA, has been ascribed to the nucleus, the β band, containing 10–15%, is associated with the chloroplast (Sager and Ishida, 1963) The origin of the γ band is not yet known If growing cells are supplied with radioactive adenine, a similar pattern appears in preparative CsCl equilibrium gradients, except that the γ band is not easily resolved from the light side of the α band, and a large amount of radioactivity which is resistant to alkaline hydrolysis appears in a pellet at the top of the gradient (Fig. 1 A). Patterns obtained when cells are grown in the presence of phosphoric acid-^{32}P were more complicated, primarily because large amounts of acid-precipitable, ^{32}P-labeled material contaminated the CsCl gradients from both top and bottom If these contaminating materials were removed from DNA-containing fractions by extensive dialysis and two or three rebandings in CsCl equilibrium density gradients, incorporation patterns similar to those found with adenine-^{3}H were revealed. If the DNA were prepared so as to minimize nucleolytic degradation, the fractions

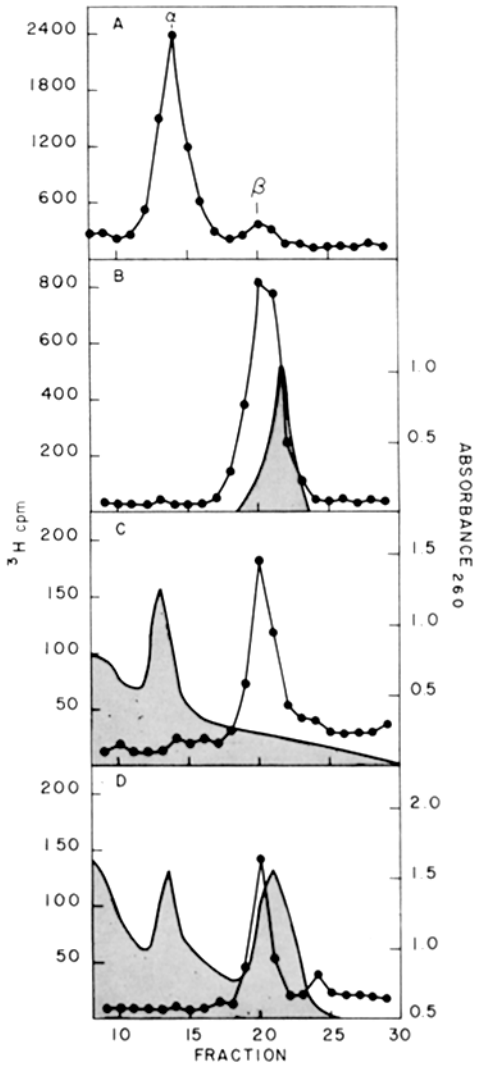

FIGURE 1 CsCl isopycnography of adenine-^{3}H and thymidine-^{3}H incorporation into *Chlamydomonas* DNA. (A) and (B) are equilibrium density gradient analyses of cells labeled with adenine-^{3}H. A culture was inoculated to a concentration of 10^4 coulters/ml with 90(−) cells and grown in the presence of 1 μCi/ml adenine-^{3}H. (A) is a whole cell crude lysate showing the clear separation of the alpha and beta peaks. In this sample, RNA has been removed from the fractions by KOH hydrolysis, and the remaining acid-insoluble material collected on membrane filters. In (B), chloroplast (β) DNA has been rebanded twice to isolate it from other labeled DNA species, and its relative buoyant density established by the addition of a *C. perfringens* (50–100 μg) density marker. Thymidine-^{3}H labeling was accomplished by growing UVS-6 cells in the presence of 2 μCi/ml thymidine-^{3}H. The result of labeling with thymidine-6-^{3}H is given in (C), and that of (methyl-^{3}H)-thymidine in (D). Both are crude lysates. A *C. perfringens* DNA marker was added to (D); note that an absorption peak corresponding to nuclear (α) DNA appears in both (C) and (D). ●—●, ^{3}H cpm; shading, absorbance at 260 nm.

containing the α band are distinctly more viscous than those containing the β band, in part because of the relative amounts of DNA in the two bands.

Thymidine Incorporation

When a growing culture was supplied with thymidine-^{3}H and the cell lysate was subsequently analyzed in a CsCl equilibrium density gradient, we found only a small fraction of the total incorporated radioactivity at the characteristic buoyant density of DNA whereas the larger fraction resided at the CsCl-mineral oil interface at the top of the gradient. The DNA labeled in this fashion forms a single peak, and Fig. 1 demonstrates that the density of this peak coincides with that of chloroplast (β) DNA. Fig. 1 B shows purified chloroplast DNA labeled with adenine-^{3}H and banded with a *Clostridium perfringens* density marker. Fig. 1 C is the result from a whole cell extract labeled with thymidine-6-^{3}H. The absorbance peak is the position of nuclear DNA; the single tritium peak indicates that incorporation occurs only into chloroplast DNA. We find a similar result when the tritium label is supplied on the methyl group of thymidine (Fig. 1 D). In this case, as in Fig. 1 B, a *C. perfringens* DNA density marker has been added to the sample just before centrifugation. The second absorption peak is again due to unlabeled nuclear DNA. The tritium peak is in the same position relative to the density marker as the adenine-^{3}H-labeled peak is in Fig. 1 B, indicating that (methyl-^{3}H)-thymidine is also incorporated into chloroplast DNA but not into nuclear DNA.

Thymidine Mono- and Triphosphate Incorporation

Labeling experiments similar to those above were repeated with thymidine-5′-triphosphate (dTTP) and thymidine-5′-monophosphate (dTMP) as the radioactive precursor. When labeled with (methyl-^{3}H)-dTTP, crude whole cell extracts subjected to CsCl equilibrium gradient centrifugation gave the pattern shown in Fig. 2 A. A similar pattern obtains when thymine-^{3}H

is used as label: neither precursor gives any indication of incorporation into RNA or DNA All of the label is found at the top of the gradient.

When cells were grown in the presence of dTMP-^{3}H, however, we did find a significant amount of incorporation specific for DNA (Fig 2 B). This pattern of DNA labeling was similar to that found when tritiated adenine (Fig 1 A) was incorporated, and it corresponded to the absorbance pattern obtained from analytical isopycnography of whole cell DNA; there is thus no evidence of preferential uptake of dTMP by either nuclear or chloroplast genomes The specific activity of the DNA labeled with dTMP

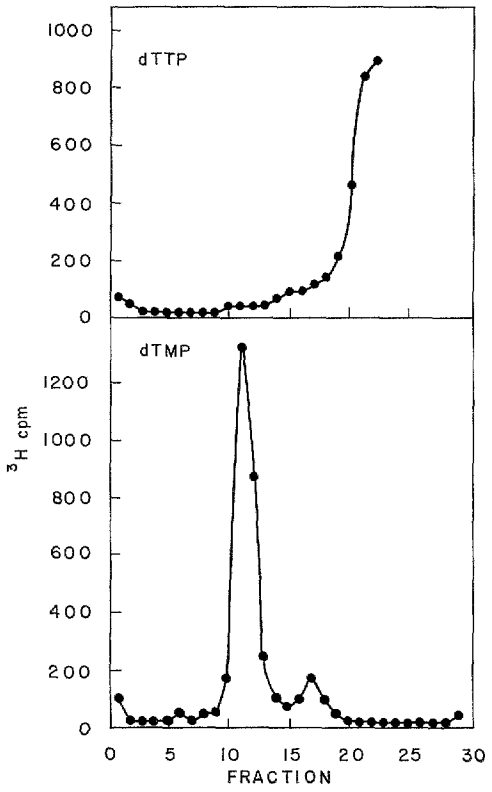

FIGURE 2 Labeling of *Chlamydomonas* with dTTP and dTMP. 10 μCi of dTTP was added to 100 ml of culture medium. The culture was inoculated with 90(−) cells to a concentration of 10^4 coulters/ml. The equilibrium density gradient of the crude lysate of this culture (upper panel) shows that there is no incorporation of tritium into DNA. dTMP labeling (lower panel) was performed with 20 μCi of dTMP under conditions similar to those used for dTTP, except that UVS-6 cells were used instead of 90(−), and only one third of the lysate was applied to the gradient.

TABLE I

Thin Layer Chromatography of Thymidine-Labeled Chloroplast DNA

Precursor	Solvent front	dCMP	dAMP	dTMP	dGMP
	cpm	*cpm*	*cpm*	*cpm*	*cpm*
(methyl-^{3}H)-dThd	220	0	56	1216	27
dThd-6-^{3}H	215	11	88	953	0

was too low by several orders of magnitude to be of value in studies on DNA replication In an experiment in which a 200 ml culture of (90-) cells was labeled with 70 μCi of dTMP-^{3}H, we determined activities of 60 cpm per μg of α DNA and 35 cpm per μg of β DNA. DNA concentration was measured by absorption at 260 nm in this experiment. We attempted unsuccessfully to increase this specific activity by altering cell permeability with dimethyl sulfoxide and simultaneously throttling the *de novo* synthesis of thymidine nucleotides with methotrexate

Chromatography of Thymidine-^{3}H Chloroplast DNA

To test whether the position at which the radioactive thymidine molecule is tritium labeled affects the pattern of DNA labeling, we determine the distribution of tritium in the chloroplast DNA of cells labeled with either thymidine-6-^{3}H or (methyl-^{3}H)-thymidine. Table I shows that 80–85 per cent of the radioactivity incorporated into chloroplast DNA was in dTMP, and that this result was independent of the position of the tritium moiety in the supplied thymidine It is likely that the 15–20 per cent of the label which traveled with the solvent front represented thymidine released from dTMP by contaminating phosphatases in the enzyme preparation

DISCUSSION

These experiments show that *Chlamydomonas* incorporates thymidine into chloroplast DNA, but not into nuclear DNA (Fig. 1). This observation confirms, by physical isolation and analysis of the chloroplast DNA, earlier observations for other algae (Steffensen and Sheridan, 1965; Chapman et al, 1966; Stocking and Gifford, 1959). The relative incorporation of tritium into chloroplast

DNA is the same whether thymidine-6-^{3}H or (methyl-^{3}H)-thymidine is supplied in the growth medium (Table I). We do observe a pellet of labeled cell debris at the top of CsCl equilibrium density gradients prepared from crude cell lysates. This makes it clear that there is probably extensive degradation of thymidine by *Chlamydomonas*, but that these degradation products are not efficiently incorporated into DNA This is not unexpected, as pathways for the degradation of thymidine have been mapped in other organisms (Fink et al , 1956 a; Fink et al , 1956 b; Hayaishi and Kornberg, 1952) Finally, the incorporation of radioactivity supplied as tritiated dTMP, while not efficient, is shown to occur into both chloroplast and nuclear DNA fractions (Fig 2). The ratio of radioactivity incorporated into nuclear DNA to that incorporated into chloroplast DNA is approximately the same whether phosphoric acid-^{32}P, adenine-^{3}H, or dTMP-^{3}H is supplied as precursor.

There is, apparently, no information yet available detailing the pathways by which *Chlamydomonas* synthesizes DNA precursors, nor is it known whether the enzymes responsible for this synthesis are located in the cytoplasm, the chloroplast, or both *De novo* synthesis of dTMP in *E coli* does not involve thymidine as a direct intermediate, but, instead, dTMP is formed by the methylation of deoxyuridine monophosphate (see O'Donovan and Neuhard, 1970 for review). If the biosynthetic routes in *Chlamydomonas* are similar to those in bacteria, one would expect dTMP to be produced *de novo* and to be incorporated into DNA. Exogenous dTMP entering the cell should suffer the same fate, providing it retains its chemical identity once inside the cell. The specific uptake of dTMP has, in fact, been observed in yeast (Jannsen et al., 1970). Our results are consistent with this possibility, although the mechanism of cellular dTMP uptake remains unexplored.

We can say, however, that the thymidine incorporation which does occur in *Chlamydomonas* is probably via direct phosphorylation of thymidine If there were extensive breakdown and reutilization by way of known pyrimidine salvage pathways, one would expect to find incorporation of radioactivity into deoxycytosine-5′-monophos phate of DNA, as well as into RNA.

If thymidine is to be used directly in DNA synthesis, thymidine kinase, which mediates the phosphorylation of thymidine, must be present. Steffensen and Sheridan (1965) have suggested that this enzyme is not present in the cytoplasm of algae, but may be controlled by the chloroplast genome. Our data are consistent with this explanation for thymidine incorporation in *Chlamydomonas*, but do not rule out, for example, the possibility that the information foı the thymidine kinase is contained within the nuclear genome, but that the enzyme itself acts within the chloroplast.

Recently, Howell and Walker (1972) showed that toluene-treated *Chlamydomonas* will incorporate dTTP-^{3}H into DNA in the presence of the other three deoxynucleoside triphosphates and magnesium. Under these conditions, 85% of the radioactivity is incorporated into DNA which has a buoyant density similar to that of *Chlamydomonas* chloroplast DNA Tritiated thymidine will not substitute for dTTP Electron microscopy of treated cells revealed that the nuclear membrane was almost totally destroyed, while the outer chloroplast membrane seemed to survive the treatment. Thus, the nuclear DNA replication system may have been dispersed but the chloroplast system may continue to function in these toluene-treated cells.

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