Regulation of Nuclear DNA Replication by the Chloroplast in *Chlamydomonas*

(replication/organelle/feedback/antibiotics)

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ABSTRACT The experiments described in this paper implicate chloroplast protein synthesis in the regulation of nuclear DNA replication. The inhibition of nuclear DNA replication in the lower eukaryote, *Chlamydomonas reinhardtii* strain 21gr, was examined after growth of cells with a series of antibiotics (streptomycin, neamine, spectinomycin, cleocin, chloramphenicol, and rifampicin) each of which has a known effect upon chloroplast RNA or protein synthesis in this organism. Each antibiotic inhibited nuclear DNA replication at drug concentrations at which there was little or no inhibition of adenine incorporation into chloroplast DNA. That chloroplast DNA was replicating under these conditions rather than merely being repaired, was shown first by the high incorporation rates and second by a ^{14}N - ^{15}N density transfer experiment in which chloroplast DNA doubled in the presence of streptomycin, while no incorporation into nuclear DNA was detected. A small DNA peak, Component III, located between nuclear and chloroplast DNA's in CsCl gradients, possibly mitochondrial, was more pronounced in DNA from antibiotic-inhibited cultures than from controls.

Mechanisms for the coordination of nuclear and organelle genetic systems are essential in eukaryotic cells (1). The tight coupling, to the cell cycle, of events in the biogenesis of chloroplasts (2) and mitochondria (3, 4) would seem to require well-synchronized, genetically controlled programs of biosynthetic activity. Evidence that this control is a two-way process with input from organelles as well as from the nucleus, is beginning to appear (5-8). These reports describe signals of organelle origin which influence nuclear gene activities required for further organelle biogenesis, i.e., feedback loops regulating organelle formation.

This paper describes the inhibition of nuclear DNA replication in the lower eukaryote *Chlamydomonas*, after growth in the presence of antibiotics that block macromolecular synthesis in chloroplasts (transcription of chloroplast RNA and protein synthesis on chloroplast ribosomes). The mechanism of inhibition is a novel one, originating in the chloroplast and regulating cell proliferation. The specificity of action of the protein synthesis inhibitors used (spectinomycin, streptomycin, neamine, chloramphenicol, and cleocin), has recently been established in *Chlamydomonas* by studies of amino acid

incorporating activity of purified chloroplast and cell sap ribosomes taken from wild-type and from drug-resistant mutant strains, in a poly(U) directed assay system (9). The specificity of action of rifampicin in *Chlamydomonas* (10) and of rifamycin SV in various systems (11) has been investigated by others.

MATERIALS AND METHODS

Preparation of Cells. Wild type *Chlamydomonas reinhardtii* strain 21gr, used in these experiments, was grown on minimal medium (12) in continuous light (15,000-17,000 lux) at 25°. Cultures larger than 100 ml were bubbled with 5% CO₂ in air.

Uniformity of the cells used as inoculum was found to be essential for reproducibility of radioisotope incorporation rates and antibiotic inhibition levels. Consequently cells were pregrown under the same conditions to be used in each of the experiments, and taken from the mid-log phase of growth. In each experiment, 100 ml cultures were inoculated with 0.5 to 1.0×10^5 cells per ml and grown 24 hr at which time the density was 1×10^6 cells per ml. Then antibiotics, and after 1 hr of incubation, $2 \mu\text{Ci/ml}$ of $2[^3\text{H}]\text{adenine}$ (New England Nuclear Corp., Boston, Mass.) were added, the flasks were incubated for an additional 23 hr, and the contents were then harvested by centrifugation and lysed immediately.

Preparation of Lysates. For lysis, each culture was resuspended in 5-10 ml of saline EDTA (0.15 M NaCl, 0.1 M ethylenediaminetetraacetate, pH 7.0), recentrifuged, and the pellet taken up into 0.7 ml of the saline-EDTA. Pronase, 0.2 ml (Pronase, Calbiochem, 100 mg/ml autodigested at 55° for 30 min and stored frozen) was added, and the cell walls digested for 10 min at 55°. The resulting sphaeroplasts were lysed with 0.3 ml of 20% sodium lauryl sulfate at 55° for 10 min. No cell walls or large cell debris could then be seen in the light microscope. Lysates were stored at -20°.

Preparative CsCl Density Gradient Centrifugation. Each lysate (1.2 ml) was transferred to a cellulose nitrate ultracentrifuge tube and 4.1 ml of stock preparative grade CsCl (American Potash Co.) solution (130 g of CsCl in 70 ml of 0.01 M Tris buffer, pH 6.8) was added. The refractive index was adjusted to 1.4000, and mineral oil added to fill each tube. Samples were centrifuged at 33,000 rpm in a Spinco 50Ti rotor for 65 hr at 19°. 20 drop fractions were collected from the bottom (there were approximately 30 fractions/tube). Each fraction was adjusted to 0.5 M KOH, digested at 65° for 2 hr or 25° for 18 hr, then cooled to 4°; 200 μg of bovine serum albumin were added, and the KOH neutralized with 20% tri-

Abbreviations: EDTA, ethylene diaminetetraacetate; TCA, trichloroacetic acid; n/t, cpm in nuclear fraction divided by cpm in nuclear + chloroplast fractions $\times 100$.

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FIG. 1. Effect of neamine on nuclear and chloroplast DNA synthesis; preparative CsCl density gradient profiles of wild type *21 gr Chlamydomonas reinhardtii* DNA which was labeled with [^3H]adenine. Cultures (100 ml) in minimal media were grown for 23 hr at 25° in continuous light in the presence of 2 $\mu\text{Ci/ml}$ of [^3H]adenine. Neamine was added 1 hr before adenine. After the cultures were harvested the spheroplasts obtained were centrifuged for 65 hr at 33,000 rpm in a 50 Ti rotor, Beckman L2-65-B ultracentrifuge at 18°. About 30 fractions (approximately 0.18 ml each) were collected through a 22 gauge, 1-1/2 inch needle from the bottom of the tube and each fraction assayed for alkali stable, cold TCA-precipitable ^3H . (A) Control; (B) 10 $\mu\text{g/ml}$ of neamine; (C) 50 $\mu\text{g/ml}$ of neamine; (D) 100 $\mu\text{g/ml}$ of neamine.

chloroacetic acid (TCA). Excess TCA was added to a final concentration of 5%. After 20 min at 4°, the precipitates were collected on Whatman GF/A glass fiber filters, washed once with cold 5% TCA, once with 95% ethanol, dried, placed in vials containing 2 ml of toluene based scintillant (liquorfluor, New England Nuclear Corp.) and counted in a Nuclear Chicago Liquid Scintillation Spectrometer.

Analytical CsCl Density Gradient Centrifugation. Analytical centrifugation was carried out in a Beckman model E ultracentrifuge equipped with ultraviolet optics. DNA was extracted and purified by a modification (13) of the Marmur method (14). DNA samples were adjusted to a refractive index of 1.4000 with optical grade cesium chloride (Harshaw Chemical Co.) and centrifuged at 44,000 rpm for 18–20 hr at 25° (15). The buoyant density of the DNA was determined by its position relative to phage SP 15 marker DNA ($\rho = 1.761 \text{ g/cm}^3$) kindly supplied by Dr. J. Marmur.

Antibiotics. Neamine, spectinomycin, cleocin, and cycloheximide were gifts from the Upjohn Co., Kalamazoo, Mich.; streptomycin was a gift from Merck & Company, Rahway, N.J. Chloramphenicol and rifampicin were purchased from Schwartz/Mann, Orangeburg, N.Y. Rifamycin SV (Na salt) was supplied by Research Laboratories Lepetit S.p.A. Milan, Italy.

RESULTS

Studies of DNA Synthesis by Radioisotope Labeling. In *Chlamydomonas*, DNA synthesis can be studied by following the incorporation of radioactive adenine into alkali stable, TCA-precipitable macromolecules. Thymidine, a preferable DNA label, is incorporated into chloroplast but not into nu-

clear DNA (16). Fig. 1A shows a typical result obtained after labeling cells for 23 hr with [^3H]adenine, lysing, extracting DNA, and centrifuging to equilibrium in CsCl.

The effectiveness of incorporation of adenine into DNA during the 24 hr experiment varied between one per 300 and one per 1000 adenine residues in controls. The two peaks of alkali stable TCA-precipitable radioactivity seen in Figs. 1 and 3 are at the positions characteristic of nuclear DNA (1.725 g/cm^3) and chloroplast DNA (1.695 g/cm^3) in CsCl gradients at equilibrium.

The ratio of cpm in the nuclear region to cpm in the nuclear plus chloroplast regions of the gradient $\times 100$ (n/t) is about 75% in Fig. 1A. A similar labeling pattern is seen as early as 4 hr after exposure to [^3H]adenine. This value is in keeping with other determinations (1) which indicate that minimal n/t is about 94% in gametes, and 76% in synchronous vegetative cultures after one round of chloroplast DNA synthesis and before the onset of nuclear DNA synthesis. (Since these cells divide into four after two rounds of DNA synthesis, average n/t of exponential cultures depends on how much the second round of chloroplast DNA synthesis overlaps the onset of nuclear DNA synthesis.) In this paper, results are reported in terms of n/t at increasing antibiotic concentrations. In preliminary experiments, the susceptibility of cells to antibiotics was found to decrease as cells approached the stationary phase. Consequently, all experiments were carried out with log-phase cells at about 1×10^6 cells per ml.

Effects of Inhibitors of Protein Synthesis on Nuclear and Chloroplast DNA Synthesis. In studies of poly(U) directed phenylalanine incorporation with purified ribosomes (9, 18), the antibiotics streptomycin, neamine, spectinomycin, chloramphenicol, and cleocin have each been shown to inhibit protein synthesis on chloroplast but not on cell sap ribosomes whereas cycloheximide was found to inhibit cell sap but not chloroplast ribosomes. The inhibitory effects of streptomycin, spectinomycin, and neamine were localized to the 30S subunit and of cleocin and chloramphenicol to the 50S subunit. In view of the effectiveness of these antibiotics at the ribosome level, they were chosen for studies of their effects on DNA synthesis.

Each drug was examined with the same experimental protocol at a series of concentrations. The results of experiments with neamine are shown in Fig. 1. With 10 $\mu\text{g/ml}$ of neamine, the incorporation into nuclear DNA decreased to about one-third of the control value, while that into chloroplast DNA remained at the control level; thus n/t fell from 76% to 40%. With 50 $\mu\text{g/ml}$ of neamine, n/t was 15% and at higher neamine concentrations, incorporation into chloroplast DNA began to be inhibited. Fig. 2 summarizes the effect of increasing neamine concentrations on n/t, and compares this result with similar experiments using spectinomycin, cleocin, and chloramphenicol. Results with streptomycin (unpublished) showed n/t values of 25% or less at all drug concentrations from 35 to 75 $\mu\text{g/ml}$ with little if any inhibition of incorporation into chloroplast DNA.

The principal finding with all five drugs was the same. Antibiotics that inhibit chloroplast protein synthesis are effective in inhibiting nuclear DNA synthesis at concentrations at which chloroplast DNA synthesis is unimpaired. Some secondary differences in the responses to these antibiotics were noted. Neamine resulted in very low n/t values at all concentrations of 25 $\mu\text{g/ml}$ and above, whereas chlor-

FIG. 2. Effect of antibiotics on adenine incorporation into DNA. n/t , cpm in nuclear fraction divided by cpm in nuclear + chloroplast fractions $\times 100$ (ordinates). The numbers on the abscissae are antibiotic concentrations. (A) neamine; (B) cleocin; (C) spectinomycin; (D) chloramphenicol. CsCl gradient experiments were as in Fig. 1.

amphenicol was effective only at concentrations of 150 $\mu\text{g/ml}$ and higher and did not result in a value for n/t of less than 50% (Fig. 2).

Since exponentially growing cultures were used in these experiments, it was expected that incorporation would continue for many hours in cells that had passed the (presumed) replication control point before the drug was added. In time-course experiments with neamine (100 $\mu\text{g/ml}$), uncoupling of adenine incorporation into nuclear and chloroplast DNA was detected as early as 4 hr after addition of the drug. No further change in n/t was noted over the subsequent 20 hr, although incorporation of adenine into both DNA's continued and leveled off between 8 and 12 hr of treatment. Only minor differences in n/t and total incorporation were seen when 8, 12, and 24 hr samples were compared. Consequently, the time in our standard experimental protocol for comparison of different drugs and different dosages was set at 24 hr.

Cycloheximide severely inhibited incorporation of adenine into both nuclear and chloroplast DNA's even at 5 $\mu\text{g/ml}$. Thus, the replication of nuclear and chloroplast DNA's is not uncoupled by cycloheximide even though at the ribosome level the drug only inhibits 80S cell sap ribosomes (18).

Fig. 3 shows two typical profiles of the effect of cleocin at 60 $\mu\text{g/ml}$ and of streptomycin at 30 $\mu\text{g/ml}$. In both profiles, the extensiveness of incorporation into the chloroplast peak is evident, as is the presence of a small peak between nuclear and chloroplast DNA's, which is more pronounced in Fig. 3A. DNA banding in this region is also seen in Fig. 1. This peak, component III, may represent a third cellular DNA, perhaps mitochondrial (see Discussion).

Effects of Rifampicin on DNA Synthesis. Rifampicin, a drug that blocks RNA polymerase activity in bacteria (11) and in the *Chlamydomonas* chloroplast (10) and a related drug, rifamycin SV, were examined for effects on DNA synthesis. With rifampicin (Fig. 4) no effect on n/t was seen below 200

FIG. 3. CsCl density gradient profiles of DNA extracted from *Chlamydomonas* cells grown with [$2\text{-}^3\text{H}$]adenine in the presence of a drug. (A) 30 $\mu\text{g/ml}$ of streptomycin; (B) 60 $\mu\text{g/ml}$ of cleocin.

FIG. 4. Effect of rifampicin (Δ) and rifamycin SV ($\square$) on adenine incorporation into DNA of *Chlamydomonas*. Data were obtained from CsCl gradient experiments. n/t , cpm is nuclear fraction divided by cpm in nuclear + chloroplast fractions $\times 100$. The numbers on the abscissa indicate the concentration of antibiotic.

$\mu\text{g/ml}$; this agrees with previous reports (10, 11). At higher drug concentrations, n/t fell to about 42%. With 125 $\mu\text{g/ml}$ of rifamycin SV, n/t was 40%. If the mode of action of rifampicin is solely upon transcription of chloroplast DNA, these results support the view that a transcription product of chloroplast DNA is required for nuclear DNA synthesis.

Density Transfer Experiments. To establish the extent of DNA synthesis, and to distinguish between replication and repair, the effect of streptomycin was examined in a ^{14}N - ^{15}N density transfer experiment. Cells, pregrown in minimal medium with $^{14}\text{NH}_4\text{Cl}$ to a titer of about 5×10^6 cells per ml, were

FIG. 5. Microdensitometer tracings of purified DNA centrifuged to equilibrium in analytical CsCl gradient. (A) Control culture grown in ^{14}N ; (B) control culture transferred to ^{15}N for 12 hr (2 generations); (C) culture incubated with 25 $\mu\text{g/ml}$ of streptomycin and ^{15}N for 12 hr. Densities of the peaks (numbers on figure) were calculated from SP 15 DNA ($\rho = 1.761 \text{ g/cm}^3$) (44,000 rpm, 20 hr at 25° in a Beckman An-F rotor).

washed with nitrogen-free medium, and resuspended at 7×10^6 cells per ml in medium containing $^{14}\text{NH}_4\text{Cl}$ and 25 $\mu\text{g/ml}$ of streptomycin. Controls were resuspended at 3×10^6 cells per ml without streptomycin. Cultures were harvested after 12 hr, DNA was extracted and purified from the three preparations (cells obtained at the start of the experiment, control cells harvested after 12 additional hr of growth, and cells harvested 12 hr after treatment) and centrifuged to equilibrium in CsCl.

Fig. 5 shows that in the presence of streptomycin, chloroplast but not nuclear DNA is synthesized; this finding confirms the results of radioisotope labeling experiments. No detectable shift in the density of the nuclear DNA occurred in the presence of streptomycin, whereas chloroplast DNA underwent approximately one doubling; and in the control culture both nuclear and chloroplast DNA's underwent two doublings.

In all of the experiments with antibiotics, the incorporation of adenine into chloroplast DNA of antibiotic treated cultures equaled that of controls (except at the highest concentrations of neamine, and spectinomycin) and the decreased n/t reflected the decreased incorporation into nuclear DNA. Thus, the likelihood that the chloroplast DNA was being replicated rather than merely repaired was strongly supported by the magnitude of the incorporation. The results of the density transfer experiment with streptomycin directly confirm the fact that under conditions of drug inhibition of nuclear DNA replication, chloroplast DNA is indeed being replicated.

DISCUSSION

The necessity for integration between nuclear and organelle genomes in eukaryotes has frequently been discussed, but almost all the evidence to date has been limited to effects of nuclear gene products upon organelle biogenesis (1). The results we report in this paper provide evidence of regulation by the cytoplasmic organelles of events directly involving nuclear biogenesis, i.e., nuclear DNA replication.

Nuclear DNA replication is inhibited by a series of antibiotics with known effects in *Chlamydomonas* on chloroplast RNA and protein synthesis. Previous studies in this laboratory have shown that neamine, spectinomycin, streptomycin, cleocin, and chloramphenicol each inhibit amino acid incorporation in a poly(U) directed system composed of purified chloroplast (70S) ribosomes, but do not inhibit at all poly(U) directed systems composed of cell sap (80S) ribosomes (9, 18). The inhibitory effects of the first three have been localized in the 30S subunit, and of cleocin in the 50S subunit (9). Rifampicin inhibits chloroplast RNA synthesis in *Chlamydomonas* by interfering with transcription of chloroplast DNA (10). Whether rifamycin SV acts similarly in *Chlamydomonas* is not known (11).

The possibility that mitochondria, rather than chloroplasts, are the primary drug target in these experiments is highly unlikely, in view of our studies of drug-resistant mutant strains. A series of cytoplasmic mutations each conferring resistance to one antibiotic (streptomycin, neamine, spectinomycin, cleocin, or carbomycin) have been shown to induce changes in chloroplast ribosomes (9, 18). In the streptomycin-resistant strain, the mutational alteration has been identified with a single protein of the 30S subunit (N. Ohta, N. Inouye, and R. Sager, manuscript in preparation). These mutant strains show no inhibition of nuclear DNA synthesis by the antibiotic to which they are resistant. Since mutational alterations in chloroplast ribosomes confer resistance to the *in vivo* effects

of the drugs, chloroplast ribosomes and not mitochondria are the primary target of drug action in *Chlamydomonas*.

Since the evidence from inhibitor studies *in vivo* is indirect, we compared the effects of several antibiotics, each at a wide range of concentration and during time course experiments. The consistency in results was shown by the inhibition of nuclear DNA synthesis whereas incorporation of adenine into chloroplast DNA was unaffected with every antibiotic tested (Figs. 2 and 4) except at the highest antibiotic concentrations. This consistency, together with our knowledge of the mode of action of these antibiotics (9, 10), provide strong support for our inference that a chloroplast gene product is an essential component in cell regulation in *Chlamydomonas* and generates a regulatory signal in the control of nuclear DNA replication.

The preparative procedures used in this study, modified from those developed for yeast (17), were devised to insure rapid and complete cell lysis, maximal extraction of DNA, and minimal manipulation of the DNA molecules. The outer cell layers are first digested with pronase and then the cells are lysed with sodium lauryl sulfate. The pronase pretreatment greatly enhances the speed and completeness of cell disruption, permitting routine recovery of over 90% of total cell DNA from log-phase cultures.

Before centrifugation, the lysate is shaken with CsCl to free the DNA from any remaining bound proteins; during centrifugation, the DNA separates from other cell constituents, and bands at its characteristic buoyant density. The effectiveness of the extraction is monitored by observing the amount of alkali stable TCA-precipitable material at the top of the gradient. Since no such material was seen in our experiments (e.g., Fig. 1A), the DNA appeared to be fully extracted.

The chloroplast DNA synthesis occurring in the presence of the antibiotics appears to be replication rather than repair. First, the magnitude of the incorporation was comparable to that of control cultures. Secondly, a density transfer experiment in which the products of DNA synthesis in the presence of streptomycin were examined in an analytical ultracentrifuge showed that all the chloroplast DNA had replicated at least once (Fig. 5).

In a few recent reports (5-8), proteins of organelle origin have been postulated to act as regulators in the production of nuclear gene products that are known to be synthesized in the cell sap, but are directly involved in organelle biogenesis. In a yellow mutant of *Chlamydomonas*, which does not make chlorophyll when grown in the dark, a major protein component of chloroplast membranes (synthesized in the cell sap) is not made when the cells are grown in the dark except in the presence of chloramphenicol (6). In *Neurospora* grown with chloramphenicol, the mycelia contain twice the control amounts of mitochondrial RNA, mitochondrial ribosomes and protein synthesis elongation factors, as well as elevated amounts of mitochondrial RNA polymerase (5). In yeast a replication factor of mitochondrial origin which indirectly affects mitochondrial DNA synthesis has been postulated because of the increased frequencies of "petite" induction after growth with chloramphenicol (7). The effect of ethidium bromide on mitochondrial DNA replication (19) may also be mediated by this factor (1). These systems differ from ours in that the feedback loops are concerned specifically with the regulation of organelle biogenesis, whereas our study has

identified a chloroplast product involved in nuclear regulation.

Our results raise the possibility that in animal cells, mitochondria may contain a feedback mechanism for regulatory control of nuclear DNA similar to the chloroplast system described in this paper.

An unexpected finding of this study was the delineation of a small DNA peak, component III, lying between nuclear and chloroplast DNA's in CsCl gradients. Component III is more pronounced in the DNA profiles from antibiotic treated cells than from controls (e.g., Figs. 1 and 3). Component III may correspond to the γ band described by Chiang and Sueoka (20) who suggested it might represent mitochondrial DNA. Our finding that component III incorporates label under conditions that inhibit nuclear DNA synthesis suggests that this component is not nuclear, and is consistent with its identity as mitochondrial. The positive identification of mitochondrial DNA of *Chlamydomonas* awaits its isolation from mitochondria.

Note Added in Proof. We recently found that neamine (50 μ g/ml) blocks nuclear DNA replication if added to synchronous cultures growing in a 12-hour light, 12-hour dark cycle, at any time up to but not after 9 hours of light, indicating that the postulated chloroplast signal is made during the first part of the light period.

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