Are Segments of Chloroplast DNA Differentially Amplified?

Jonathan R. Mielenz and Charles L. Hershberger
Department of Microbiology, University of Illinois, Urbana, Illinois 61801

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Five species of covalently-closed circular DNA were identified in the chloroplast fraction of DNA from Euglena gracilis. These components exhibit buoyant densities corresponding to fractions that are separated by fragmentation of chloroplast DNA. It is suggested that these covalently-closed circular molecules may reflect amplification of particular segments in chloroplast DNA.

Chloroplasts of Euglena gracilis contain covalently-closed circular (ccc)*DNA with a molecular weight of 92 x 10^6 daltons and a buoyant density of 1.685 g/cm^3 (1). The chloroplast DNA (cDNA) can be fragmented to yield components with buoyant densities of 1.684 (2), 1.687 (2), 1.692 (2), 1.696 (3) and 1.700 g/cm^3 (2,3,4). In addition Nass and Ben-Shaul (5) have isolated from Euglena a cccDNA with a molecular weight of 6 x 10^6 daltons and a buoyant density of 1.700 g/cm^3.. When heterotrophically grown cultures of Euglena undergo the transition from exponential growth to stationary phase, species of DNA with buoyant densities of 1.692 g/cm^3 and 1.700 g/cm^3 undergo preferential replication (6).

The above observations suggest a unique model for the structure and replication of cDNA in Euglena (Figure 1). cDNA contains segments with nucleotide compositions different than the average nucleotide composition of the entire molecule. Depending on the size of fragments, segments with different nucleotide compositions, therefore buoyant densities, are separable. During particular stages in the life cycle these segments are preferentially amplified. The model assumes that the amplification products are cccDNA's.

In order to verify the model it is necessary to demonstrate that the hypothetical amplified components exist as separate molecules. This paper

*Abbreviations: ccc, covalently-closed circular; nDNA, nuclear DNA; cDNA, chloroplast DNA; PrI, Propidium iodide; L-, lower buoyant density in equilibrium density gradients; H-, higher buoyant density in equilibrium density gradients; rRNA, ribosomal RNA.

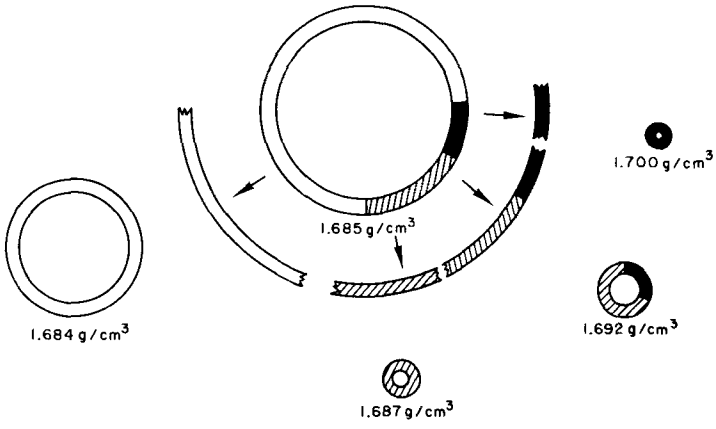

Fig. 1: Model for the Structure of Chloroplast DNA

The figure schematically represents the structure of cDNA. The entire cDNA molecule is circular with a buoyant density of 1.685 g/cm^3. Segments of the chloroplast DNA are designated by the solid section, hashed section, and plain section of the circular molecule. After shearing the entire cDNA, fragments representing each segment have been identified as listed in the text. The drawing is schematic and does not imply that all of the segments are simultaneously identified after shearing to a single fragment size. Segments are differentially amplified when the culture undergoes the transition from exponential growth to stationary phase. Amplified segments are indicated by the smaller circles. Buoyant densities of the circular DNA's and fragments corresponding to the same segment of cDNA are indicated under the circles.

reports results from initial experiments to test the hypothesis. DNA was isolated from cultures during the transition from exponential growth to stationary phase and five species of cccDNA were identified by equilibrium density gradient ultracentrifugation in the presence of propidium iodide (PrI).

MATERIALS AND METHODS: Euglena gracilis strain Z was grown in heterotrophic medium with 3.5 g/l of succinic acid as a sole carbon-source (8). Cultures were grown under steady-state labeling conditions with 50-100 μCi/ml of ^{32}P PO_4. DNA was isolated as described by Gibson and Hershberger (6), except the acetone extraction and alcohol precipitation steps were normally omitted. Procedures for ultracentrifugation and collection of fractions from the top of the gradient have been described by Stolarsky et. al. (9). Aliquots for liquid scintillation counting were spotted on Whatman 3MM paper discs. The

discs were washed three times with 5% trichloroacetic acid, two times with 70% ethanol, and once with ethanol-ethyl ether (1:1) and air dried. Samples containing PrI were washed three times with cold 2-propanol instead of 70% ethanol. Filter discs were counted in cocktail containing 0.1 g of 2,2'-p-phenylenebis (5-phenyloxazole) and 4.0 g of 2,5-diphenyloxazole in 1.0 l of toluene. Reference DNA's were isolated as described by Marmur (10) from: Micrococcus lysodeikticus (1.731 g/cm^3), Serratia marcescens (1.718 g/cm^3), Escherichia coli (1.710 g/cm^3), Proteus mirabilis (1.699 g/cm^3), and Bacillus cereus (1.695 g/cm^3). Nuclei were isolated from Tetrahymena pyriformis (11) and DNA (1.684 g/cm^3) was prepared by Marmur's procedure (10).

EXPERIMENTAL RESULTS: DNA was isolated from cultures in the transition between exponential and stationary phases of growth. The DNA was separated into light (L) and heavy (H) fractions (Figure 2). Re-examination by ultracentri-

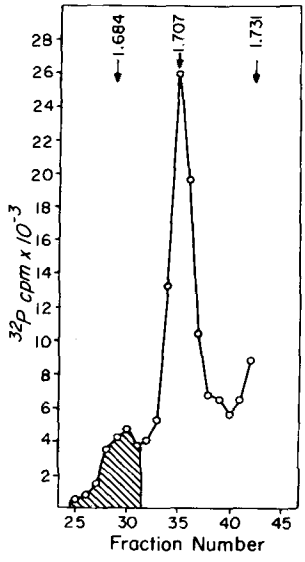

Fig. 2: Equilibrium Density Gradient Ultracentrifugation of Whole Cell DNA

Cells were harvested during the transition from exponential growth to stationary phase and DNA was isolated and centrifuged to equilibrium as described in Materials and Methods. Fractions in the cross-hatched area were pooled as the L-fraction and employed for subsequent analyses. The arrows designate the A_{260} positions for marker DNA's as indicated in Materials and Methods.

fugation indicated that the L-fraction contained approximately equal quantities of cDNA and nuclear DNA (nDNA) with buoyant densities of 1.685 g/cm^3 and 1.708 g/cm^3. Profiles of the two components exhibited considerable overlap similar to that seen in Figure 2.

In order to identify cccDNA the L-fraction was centrifuged to equilibrium in CsCl-density gradients containing PrI (Figure 3). Ultracentrifuga-

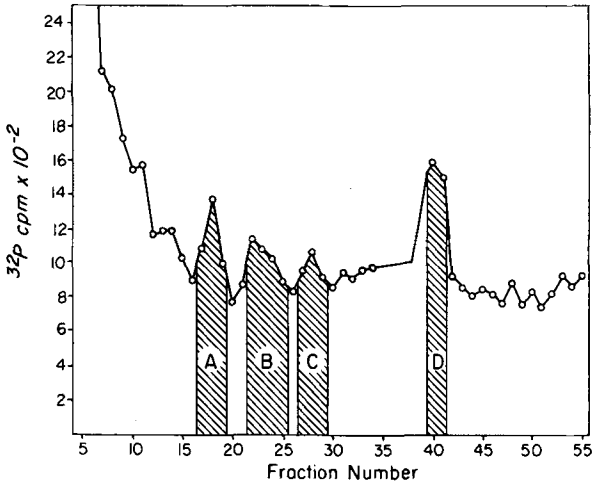

Fig. 3: Equilibrium Density Gradient Ultracentrifugation of L-Fraction DNA in the Presence of PrI

The L-fraction from Figure 2 was centrifuged to equilibrium in a CsCl gradient containing 400 µg/ml PrI. The cross-hatched areas designated A-D were separately pooled and employed for subsequent analysis.

tion of the fractions near the top of the profile in Figure 3 indicated that these fractions contained mostly nDNA with a buoyant density of 1.708 g/cm^3 and some cDNA with a buoyant density of 1.685 c/m^3. Although cDNA and nDNA have different buoyant densities, the relaxed forms of these DNA species were not resolved near the top of the gradients containing PrI (Figure 3). Four peaks (A, B, C and D in Figure 3) were seen at buoyant densities expected for cccDNA (12). Fractions in these four peaks were separately pooled and extracted with 2-propanol to remove PrI as previously described (7). Explana-

tions for the high backgrounds of radioactivity in the gradients containing PrI were not determined; however, higher ratios of peak height to background have been obtained in more recent experiments. Isolation of a distinct species of DNA from each peak (discussed below) and re-examination by ultracentrifugation with PrI verified the assignments of A-D in Figure 3.

Each of the fractions, A-D, were centrifuged to equilibrium in CsCl-gradients without PrI (Figure 4). The profiles in Figure 4 exhibited skewing toward heavier buoyant densities; however, the known effects of viscosity on fractionation of DNA precluded the interpretation that the components were heterogenous. Buoyant densities of the major species of DNA were 1.690 g/cm^3 for A, 1.686 g/cm^3 for B, 1.690 g/cm^3 for C and 1.686 g/cm^3 for D. These experiments identified four distinct species of cccDNA with buoyant densities between 1.684 and 1.692 g/cm^3, as predicted by the model in Figure 1.

Experiments in Figures 2-4 have been independently repeated six times; however, slightly different results were obtained with a culture that had completed the transition from exponential growth to stationary phase. The DNA was isolated and separated into L- and H-fractions with results similar to those in Figure 2. The L-fraction was examined for cccDNA as described above. The results in Figure 5 demonstrate three components: one at the position of fraction B, one at the position of fraction D and a new component seen at position E with a greater buoyant density than fraction D. Each of these components was examined by ultracentrifugation without PrI. The results in Figure 6 indicate that fraction B contained DNA with a buoyant density of 1.684 g/cm^3 while fractions D and E each contained two components with buoyant densities of 1.685 and 1.699 g/cm^3. These results are consistent with existence of the fifth species of cccDNA that was predicted by the model in Figure 1.

DISCUSSION: The hypothesis in Figure 1 proposes that cDNA from Euglena contains three segments with nucleotide compositions that differ from each other and differ from the average nucleotide composition of the entire molecule, therefore, the segments exhibit different buoyant densities. The model also

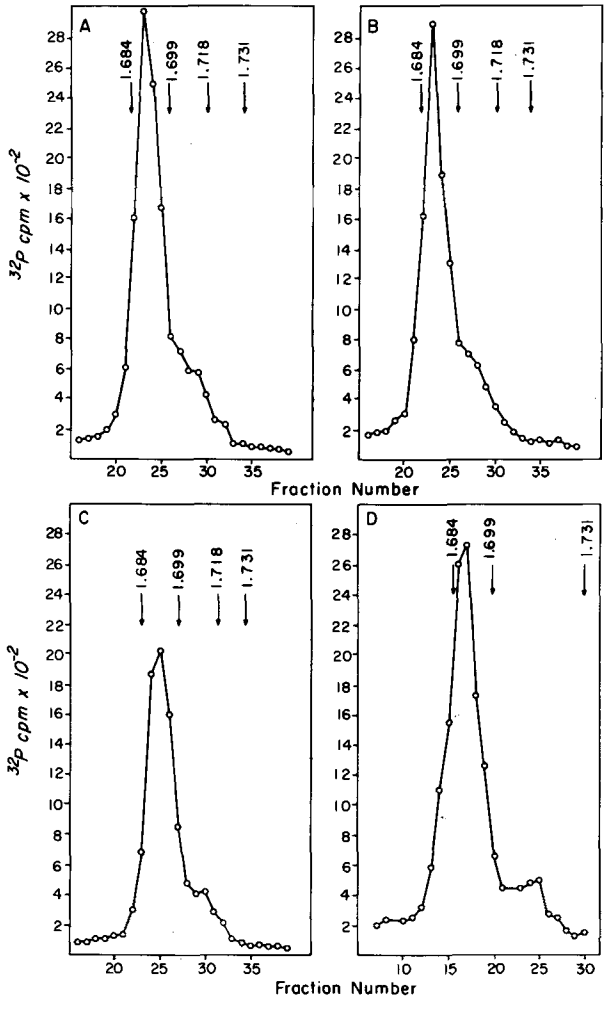

Fig. 4: Equilibrium Density Gradient Ultracentrifugation of Separate ccc Components

Fractions A-D from Figure 3 were separately centrifuged to equilibrium in CsCl-gradients. The alphabetical lettering of the figures corresponds to the alphabetical designation of the pooled-fractions in Figure 3. The arrows designate the A_{260} positions for marker DNA's.

proposes that different segments and combinations of segments are preferentially amplified at different stages of growth to produce four species of cccDNA in addition to ccc molecules of the complete cDNA. The amplification products represent the segments with buoyant densities of 1.684, 1.687 and

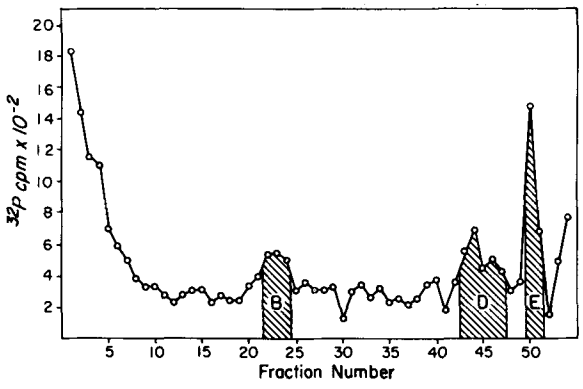

Fig. 5: (Top) Equilibrium Density Gradient Ultracentrifugation of L-Fraction DNA in the Presence of PrI.

Cells were harvested from a stationary phase culture and DNA was isolated as described in Materials and Methods. The L-fraction was isolated as described in Figure 2 and employed for equilibrium centrifugation in a CsCl gradient containing 430 µg/ml PrI. Cross-hatched areas were separately pooled and correspond to fractions with the same designations in Figure 3.

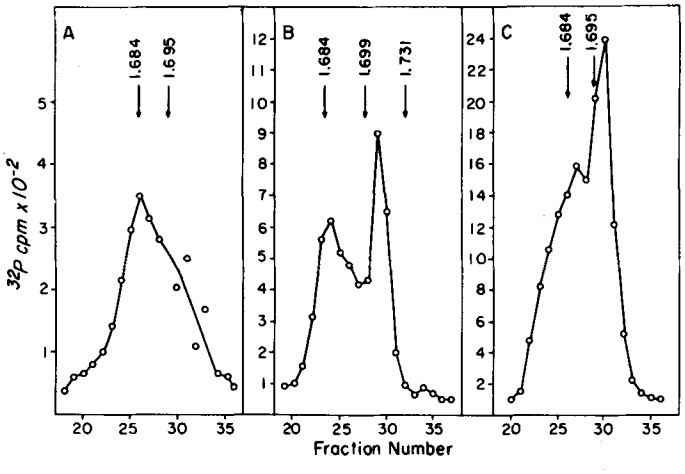

Fig. 6: (Bottom) Equilibrium Density Gradient Ultracentrifugation of Separate ccc Components.

Fractions B, D, and E from Figure 5 were separately centrifuged to equilibrium as described in the legend to Figure 4.

1.700 g/cm^3 in addition to a composite segment with a buoyant density of 1.692 g/cm^3 and containing the segments with buoyant densities of 1.700 and 1.687 g/cm^3. If the molecular weight of the 1.700 g/cm^3 segments is taken as 6 x 10^6 daltons (5) molecular weights of the other components are estimated as

12 x 10^6 daltons for the 1.687 g/cm^3-segment and 74 x 10^6 daltons for the 1.684 g/cm^3-segment.

Results of this investigation demonstrate the existence of five different species of cccDNA in Euglena. Within the sensitivity of measuring buoyant densities the components also exhibit the buoyant densities hypothesized in the model. The model should be considered tentative until it is substantiated by more rigorous criteria. It is critical to test the homology between the species of cccDNA and the complete cDNA. The correlation between the buoyant densities of cccDNA's and various fragments of cDNA is circumstantial. Specifically, mitochondrial DNA with a buoyant density of 1.688 g/cm^3 (13) has not been accounted for in these experiments. Numerous attempts, however, have failed to identify ccc mitochondrial DNA (personal communications from O. Richards and M. M. K. Nass). The separations effected by PrI indicate that the species of cccDNA differ in superhelix densities as well as buoyant densities. Several explanations for similar results with intercalating dyes have been documented (14,15,16,17). More extensive structural investigations are required to explain these observations in this investigation.

The model is indirectly supported by six types of observations. When heterotrophically grown cultures of Euglena reach stationary phase the chlorophyll content of the cells increases dramatically (18). The cistrons of chloroplast rRNA are enriched in the fragments with buoyant densities of 1.692 (2), 1.696 (3) and 1.700 g/cm^3 (2,3,4) and depleted in the fragments with buoyant densities of 1.684 and 1.687 g/cm^3 (2). More extensive fragmentation of the component with a buoyant density of 1.692 g/cm^3 yields fragments with buoyant densities of 1.687 g/cm^3 and 1.703 g/cm^3 (2). Chloroplasts isolated from cultures in different stages of growth appear to contain different numbers of cistrons for rRNA (3,4). Up to 1/3 or 1/2 of the cDNA has a buoyant density of 1.700 g/cm^3 during exponential growth of photosynthetic cultures (19), whereas this component is not detected during exponential growth of mixotrophic cultures (6). When DNA from exponential cultures is examined as

described above, fraction B is the only detected species of cccDNA (unpublished observations).

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