EXTRANUCLEAR DNA IN POLYOMELLA PARVA

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology by Karen B. Darnall

January, 1986
The thesis of Karen B. Darnall is approved:

Joyce B. Maxwell

Richard L. Potter

Kenneth C. Jones, Chairman

California State University, Northridge
ACKNOWLEDGMENTS

I wish to thank the members of my committee for their support and guidance in the preparation of this thesis. I would also like to thank Dr. Marvin Cantor, Dr. Clifford Brunk, and Dr. Michael Mann for their suggestions and technical advice. I am also grateful to Gayle Pomraning and Carolyn Shuldig for their many hours of assistance in the laboratory.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Isolation Techniques</td>
<td>4</td>
</tr>
<tr>
<td>Characterization of the DNA</td>
<td>7</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>10</td>
</tr>
<tr>
<td>Cell Culture</td>
<td>10</td>
</tr>
<tr>
<td>DNA Isolations</td>
<td>10</td>
</tr>
<tr>
<td>Southern Blot Hybridization</td>
<td>12</td>
</tr>
<tr>
<td>Fragment Isolations</td>
<td>12</td>
</tr>
<tr>
<td>Labeling and Purification of the Probe</td>
<td>13</td>
</tr>
<tr>
<td>Southern Transfer</td>
<td>14</td>
</tr>
<tr>
<td>Prehybridization and Hybridization</td>
<td>14</td>
</tr>
<tr>
<td>DNA Characterization</td>
<td>17</td>
</tr>
<tr>
<td>DNase Treatment</td>
<td>17</td>
</tr>
<tr>
<td>Exonuclease Treatment</td>
<td>17</td>
</tr>
<tr>
<td>Mitochondrial Preparations</td>
<td>19</td>
</tr>
<tr>
<td>I.D. of DNA in the Mitochondrial Fraction</td>
<td>21</td>
</tr>
<tr>
<td>Mitochondrial Isolation on Sucrose Gradient</td>
<td>21</td>
</tr>
<tr>
<td>Succinic Dehydrogenase Assay</td>
<td>21</td>
</tr>
<tr>
<td>DNA Extraction</td>
<td>22</td>
</tr>
<tr>
<td>Molecular Weight Estimation of the 3.7 kb Band</td>
<td>24</td>
</tr>
<tr>
<td>RESULTS</td>
<td>25</td>
</tr>
<tr>
<td>Southern Blot Hybridization</td>
<td>25</td>
</tr>
<tr>
<td>DNase Dilution Series</td>
<td>26</td>
</tr>
<tr>
<td>DNA Characterization with Enzymes</td>
<td>28</td>
</tr>
<tr>
<td>Mitochondrial DNA Preparations</td>
<td>29</td>
</tr>
</tbody>
</table>
Succinic Dehydrogenase Activity .......................... 30
DISCUSSION .................................................. 31
REFERENCES .................................................. 38
ABSTRACT

EXTRANUCLEAR DNA IN POLYtomELLA PARVA

by
Karen Brower Darnall
Master of Science in Biology

When DNA was extracted from the phytoflagellate P. parva, and subjected to electrophoresis, a prominent low molecular weight band appeared at a position corresponding to 3.7 kb. The DNA was shown to be associated with mitochondrial membrane fragments and most likely exists as mitochondrial plasmids, common to many plants and fungi. The DNA molecule appears to be linear, and its susceptibility to exonuclease digestion indicates that there are no telomeres present. A Southern blot hybridization was performed with an enzymatic labeling system. The fragment hybridized with itself, but not with DNA in other portions of the gel, suggesting that the sequence of the element is not present in the larger DNA fragments.
INTRODUCTION

Polytomella parva is a fresh-water alga that has been studied intensively at California State Northridge. The organism multiplies rapidly and is easily cultured in a simple broth medium at room temperature. A culture was obtained from the protozoology laboratory for an experiment requiring a large amount of eukaryotic DNA. The first sample of DNA was extracted and examined for purity and intactness by agarose gel electrophoresis. A sharp, distinct, low molecular weight band was visible and it migrated well past the main chromosomal smear. Molecular weight markers indicated a size of approximately 4 kilobases.

The first objective of this project was to find the source of the small DNA fragment in *P. parva*. Was it membrane-bound or free in the cytoplasm? Was the DNA in an organelle, and if so, which one? Several possibilities existed as a possible source of the DNA: *P. parva* includes a single nucleus, one or more mitochondria, and a few plastids and proplastids. Cytoplasmic sources of DNA have also been found in other protozoa in the form of microorganisms. One species of *Paramecium* has been shown to contain various types of Gram-negative bacteria, and another harbors an endosymbiotic alga (Goodenough, 1984). Viral particles are also commonplace in many eukaryotes.
After considering all of the possible sources of DNA, a decision was made to start with a mitochondrial preparation. The difficulty of this technique was not anticipated, however. Attempts to isolate intact mitochondria were not successful, but mitochondrial fragments that appeared to be free of chromosomal DNA yielded the 4 kb fragment as well as two other bands at approximately 20 kb.

A second objective was to characterize the fragment. It was not certain, until the fragment was labeled with biotinylated dUTPs by nick-translation, that the band consisted of DNA and not RNA. Since the 4 kb DNA, that had been extracted from low melting temperature agarose, was not easily obtained in quantity, other experiments were performed without separating the fragment from the rest of the DNA. Total *P. parva* DNA was subjected to a variety of digestive enzyme treatments and electrophoresed. Since the fragment was so prominent, it was possible to distinguish the changes in electrophoretic patterns without much interference from the high molecular weight DNA. A Southern blot hybridization with the labeled fragment was used to detect homologous sequences that might be repeated within the larger DNA fragments.

A survey of the literature revealed no previous publications concerning DNA in *P. parva*. Extensive electron microscopic examinations had revealed many of the fine morphological details of the organism, and other papers
described physiological traits.

Polytomella was initially described by Arago, who established the organism as a new genus in 1910. He named it after Polytoma, a similar alga. Polytomella is nearly spherical, enclosed by a thin membrane, and has four flagella. It swims very rapidly, frequently changing direction and sometimes spinning in place. It exists as a single cell, varying in size by a factor of two, with a mean diameter of 10μm. The mitochondrial area is at the anterior end where the flagella arise from four separate basal bodies. The nucleus is centrally located, and plastids, proplastids, Golgi bodies, and other dense vesicles can be found scattered throughout the cell. The cells accumulate numerous membrane-bound starch granules which obscure the other organelles when viewed with a light microscope.

There is some question as to the number of mitochondria per cell. Burton and Moore, 1974, made three-dimensional scale models of mitochondria from three different individual cells on the basis of electron micrographs. One individual was found to have a single mitochondrion, and others had 4 to 10. It is possible that the greater numbers may have been due to artifactual breakage. The single mitochondrion was basket shaped and occupied approximately 19% of the cell's total volume. The mitochondrion may also lend some structural support to the cell (Burton and Moore, 1974).
Isolation Techniques

Two mitochondrial isolation techniques involving homogenizing and sonifying the cells proved too harsh, rendering small membrane fragments instead of intact organelles. A gentler procedure which was originally used for the isolation of trypanosomal amastigotes was found. The amastigotes are similar to P. parva with their thin membranes and spherical shapes. Lysis was achieved by suspending the cells in a hypotonic solution. The procedure was modified by experimenting with buffers of various ionic strengths, pH, and temperatures. Wet mounts were made periodically with Janus green stain (an indicator specific for the oxidizing enzymes of mitochondria) in order to monitor the lytic process. Large amounts of blue debris gave evidence that the mitochondria were bursting. DNA isolations were made from preparations that appeared to include dense particles and a small amount of debris.

Since mitochondrial DNA had not been reported from this organism, it was necessary to demonstrate that our technique was successful. Electron micrographs of the mitochondrial pellet are usually used to demonstrate the characteristic cristae. This was not done in this investigation. Rather a correlation was sought between the 4 kb band and succinic dehydrogenase activity of the material from which it was extracted.
Before mtDNA is isolated, the mitochondria are normally treated with DNase. The enzyme digests nuclear DNA clinging to the outer membranes, but more importantly, it verifies that the DNA subsequently recovered must have been protected by intact membranes during the incubation. Unfortunately the mtDNA of *P. parva* was repeatedly destroyed by this process. Mitochondrial isolations from untreated preparations consistently produced two distinct sizes of DNA, however. The larger fragment of 20 kb was similar in size to the mtDNA found in a closely related organism, *Chlamydomonas* (Ryan et al. 1978). This similarity prompted further attempts to demonstrate that the 4 kb band was mitochondrial in origin.

It was assumed that at least some of the mtDNA would cling to the mitochondrial membranes. In fact, the convoluted inner membrane surface would seem likely to trap large amounts of DNA. Presumably, any amount of nuclear DNA contamination would be negligible, in comparison to the mtDNA extracted from the mitochondrial pellets.

Additional purification of mitochondrial fragments was accomplished by sucrose gradient centrifugation. The central portion of the gradient contained material that was high in succinic dehydrogenase activity. This portion also produced the 20 kb and 4 kb fragments, indicating that the DNA was extracted from material rich in a mitochondrial enzyme. Furthermore, the lowest fraction of the gradient, which normally collects unbroken nuclei
(Cantor and Sheeler, 1970), produced some high molecular weight DNA and none of the 4 kb fragment.
Characterization of the DNA

A few simple experiments were devised to learn as much as possible about the fragment. The first experiment was a more careful measurement of the fragment, and the size was estimated at 3.7 kb by agarose gel electrophoresis.

Another test was made possible by the recent availability of enzymatic DNA detection systems. Although the biotinylated probes are not as sensitive as radioactively labeled DNA, they hybridize just as specifically, and they can be obtained without a special license (Murasugi and Wallace, 1984). The 3.7 kb band was removed from a low melting point agarose gel, labeled, and used to hybridize with total P. parva DNA on a Southern blot. The fragment bound only to itself, indicating that the sequence is unique to the 3.7 kb band.

There are various means of determining the forms of DNA fragments. The most informative is that of an electron micrograph of a purified specimen. A less complicated strategy was used to gain some insight into the conformation of the molecule, based on the differences of electrophoretic mobilities of disparate forms of DNA. When DNA is electrophoresed through an agarose or polyacrylamide gel, the migrational rates are inversely proportional to the frictional coefficients, which depend on the size and shapes of the molecules. Circular DNA
elements can exist in three different conformations: loose, open circles; compact, supercoiled circles; or linear molecules. The open circular DNA migrates most slowly because it offers more resistance than the supercoil; and it cannot "snake" its way through the gel matrix as the linear strand can. The relative mobilities of the linear and supercoiled molecules depend on electrophoretic conditions, but they generally migrate at different rates (Rodriguez and Tait, 1983).

Total DNA extracts from *P. parva* were subjected to different degrees of digestion by DNase I. If the 3.7 kb band had consisted of supercoiled molecules, low concentrations of the enzyme would have nicked the strands, causing the supercoil to relax and assume an open circular form. Higher concentrations of the enzyme would have linearized the molecule, and very high concentrations would have digested away all the material. The experiment showed no change in the mobility of the 3.7 kb band at any of several DNase concentrations. This suggests that the molecule is linear.

The mechanism by which the ends of linear DNA replicate is a problem that is solved differently by various organisms. DNA polymerase synthesizes complementary DNA strands starting from an RNA primer and proceeds only in the 5' to 3' direction. Excision of the RNA primers leave gaps. The gaps can be avoided by the formation of circular intermediates which are subsequently cleaved.
Another solution to the problem is to link the two DNA strands together at the ends so that a hairpin loop is formed. These structures are called telomeres and are found at the ends of eukaryotic chromosomes as well as the termini of linear yeast plasmids.

A common method of determining whether the ends of a linear molecule are blunt-ended or not, is by attempting to replace the terminal phosphates with $^{32}\text{P}$. The enzymatic system utilizes labeled nucleotides, not phosphates, so this method was not feasible. An alternative procedure made use of a digestive enzyme, exonuclease III, which requires free 3'OH groups for activity. It catalyzes the release of 5' mononucleotides from the 3' end of duplex DNA, but is also able to cleave apurinic or apyrimidinic sites (New England Biolabs 1983-84). The enzyme was able to digest the band, which indicates that the 3'OH groups are present, but does not rule out the possibility of gaps within the strands.
MATERIALS AND METHODS

Cell Culture

*P. parva* was grown at room temperature in carboys containing seven liters of a complex broth: 0.1%(w/v) tryptone, 0.2%(w/v) yeast extract, and 0.2%(w/v) sodium acetate (Moore et al. 1970). Air was passed through a sterile filter and bubbled through the medium. At room temperature, it took four to five days for the cells to reach the stationary growth phase. Inocula consisted of cells grown in 100 ml of the same medium without bubbling air. Chloramphenicol (50 μg/ml) was added to the carboy at the time of inoculation to prevent bacterial contamination. Cells were stained with Kova stain (ICL Scientific) and counted in a hemocytometer chamber before they were harvested. Cell counts generally ranged from 2 to 3 x 10⁶/ml.

DNA Isolations

When the cells were deprived of air at the stationary growth phase, they tended to agglutinate and settle to the bottom. When the carboy was tipped at a 45° angle, the cells formed a pellet in approximately one hour, and the broth was siphoned off the top.

The pellet was washed once with distilled water,
then immediately suspended in 100 ml of 60°C NET buffer (0.5 NaCl, 0.05 M EDTA, 0.05 M Tris-8.5) with 0.1% Sarkosyl. The high concentration of salt and the heat were used to inactivate DNases I and II normally found in eukaryotic cells (Brunk, personal communication).

The mixture was incubated at 60°C for one hour with frequent agitation. Protein and starch were extracted three times with a 24:1 chloroform/n-amyl alcohol solution. The aqueous layer was dialyzed overnight to reduce the salt concentration and then extracted once again with phenol/chloroform. The nonpolar residue was removed with ether. DNA was then precipitated by adding one-tenth volume 3 M sodium acetate and two volumes of ice-cold isopropanol. The pellet was redissolved in RNase buffer (20 μg/ml RNase, 0.5 mM NaOAc).

The same procedure was followed for the mitochondrial DNA isolations; however, when the mitochondrial fractions were taken from the sucrose gradient, five volumes of isopropanol were needed instead of two because of the residual sucrose in solution (Maniatis et al. 1983).

The DNA was checked to make certain that the molecular weight was high and that the RNA was digested by electrophoresis on a 0.5% agarose gel. An additional well was loaded with Hind III-digested lambda DNA fragments which provided a molecular weight standard. Tris-acetate buffer, pH 8.0, was used for all gels and electrophoresis.
Southern Blot Hybridization

**Fragment Isolation**

The band of homogeneous DNA was isolated by extraction from a melted agarose slice. A preparative mini-gel was made with 0.5% low melting temperature agarose. Tape was applied to the comb so that one well and one long trough would be formed in the gel. Buffer and gel were chilled to 4°C before use. 200 µl of *P. parva* DNA was loaded in the trough and lambda Hind III-digest was loaded in the marker well. DNA was electrophoresed for three hours at 60 volts and then stained with ethidium bromide. The band was visualized by UV illumination and cut out with a razor blade. The slice was turned on its edge and excess agarose was removed.

The gel was melted in an eppendorf tube at 65°C. Two volumes of 65°C buffer C (50 mM Tris-8.0, 10mM NaCl, 10 mM EDTA) were added, mixed well, and incubated an additional half hour. An equal volume of 65°C phenol was used to extract the agarose. After the phenol was added, the tube was shaken vigorously for three minutes. The upper phase was removed after centrifugation, with care being taken not to remove the interface. Phenol was removed by two chloroform extractions, and the chloroform was removed with ether. The DNA was precipitated overnight at -20°C (Perbal 1984). A large portion of the isolated
DNA was made directly into a biotinylated probe, and a smaller portion was restricted and cloned into pUC-19 for later use.

**Labeling and Purification of the Probe**

The BRL nick-translation system was used to label the DNA with biotin according to the instructions in the product insert. 3 μg of plasmid DNA was labeled, and 200-300 ng of DNA from the gel was labeled with biotin-11-dUTP (BRL).

Biotinylated DNA was separated from free nucleotides by filtration over a Sephadex G-50 column, using a 5 ml plastic pipette. The tip was packed with siliconized glass wool, and a two inch length was cut from the top. The column was packed with four and one half mls of resin and equilibrated with 1 X sodium chloride-sodium citrate, 0.1% SDS eluting solution. The 50 μl nick-translation mixture was loaded on the top of the column, and after it was absorbed, an additional 200 μl of eluting solution was used to rinse the reaction tube, and was added to the column.

Fractions were collected in 20 tubes. The first tube contained 1 ml of eluting solution, and 150 μl was collected in each of the remaining tubes. 2 μl aliquots were taken from each tube and spotted on nitrocellulose paper. The paper was developed with the BRL Biotin Detec-
tion System according to the instructions, but it was found that the filter blocking step with BSA could be eliminated for the purpose of isolating the probe. DNA was generally detected in 3 or 4 of the tubes in the middle of the series.

Southern Transfer

A "Baby Gel" apparatus (BRL H6) was used to electrophorese a sample of *P. parva* DNA, lambda-Hind III marker, and *Tetrahymena thermophila* DNA. The 1.1% agarose gel was run at 60 V for one and one half hours.

Nitrocellulose paper, wicking sheets, and blotting pads were contained in the baby blot kit and were used for the transfer. The gel was depurinated for 5 minutes in 3 M HCl and rinsed with distilled water; the DNA was denatured and then neutralized according to the instructions supplied with the kit. The gel was blotted overnight, and the nitrocellulose was baked for two hours in an 80°C vacuum oven.

Prehybridization and Hybridization

Prehybridization treatment and hybridization conditions (Leary et al. 1983) were recommended by BRL. Nitrocellulose paper was prehybridized in 10 ml of 50% formamide, 5 X SSC, 5 X Denhardt's solution, 25 mM sodium
phosphate pH 6.5, and 250 µg of salmon sperm DNA. The salmon sperm DNA was sheared by forcing a 4 mg/ml solution through a 25 gauge needle several times (Perbal 1984). The sheared DNA was boiled for 10 minutes and chilled in an ice-ethanol bath before it was added to the hybridization mixture.

The nitrocellulose paper and prehybridization solution were sealed in a Dazey Seal-a-Meal bag and incubated for 4 hours at 65°C.

The hybridization solution had the same ingredients except for the addition of 10% dextran sulfate and the reduction of formamide to 45%. Probes for the 3.7 kb band and the lambda-Hind III marker were denatured in the same manner as the sperm DNA and added to the prewarmed (42°C) hybridization solution. Both filter and solution were put into a new bag and bubbles were carefully eliminated before sealing. Hybridization was allowed to continue for 24 hours at 42°C.

Post-hybridization rinsing steps were recommended by BRL in the detection kit instructions. The paper was washed seven times with SSC, SDS solutions, including two incubations at 50°C. The paper was not dried before developing.

Biotinylated DNA was detected by the streptavidin, alkaline phosphatase system made by BRL. The reactions and washes were performed according to the instructions provided by BRL, and the filter blocking step was included.
The last reaction, precipitation of the colored substrate, was processed in a Sears sealing bag, because the nylon component in most other bags reacts with the substrate and causes a high background (BRL Focus 1985).
**DNA Characterization**

**DNase Treatment**

1 mg of DNase I (Sigma) was reconstituted in 1 ml of buffer (0.2 M NaCl, 5 mM MgCl₂, 10 mM sodium acetate, 0.5 mg/ml nuclease free BSA). Purified plasmid DNA was tested in order to demonstrate the change in the electrophoretic pattern of the DNA with gradual nicking by DNase.

A 1:2 dilution series of DNase was carried through eight tubes starting with 0.5 μg/ml. Each dilution was made in 5 μl of the same buffer as the original stock. A 15 μl cocktail of 0.06 μg plasmid DNA and core buffer (BRL) were added to each tube. The reaction mixtures were incubated at 37°C for 45 minutes. Half of each mixture was loaded on an agarose gel and electrophoresed at 60 V for one hour (Mann, personal communication).

The same experiment was repeated with *P. parva* DNA. The first tube of the dilution series contained 0.1 μg/ml DNase, and one lane was used to show undigested DNA. Another dilution series was made with Eco RI in water. The initial tube contained 0.4 units of enzyme.

**Exonuclease Treatment**

*P. parva* DNA was treated with exonuclease III (BRL) to test for the presence of exposed 3'OH groups. A low
salt buffer was used (66 mM Tris-7.5, 66 mM MgCl$_2$, 6.5 mM NaCl, 5 mM DTT), and the DNA was digested for one hour at 37°C. Electrophoresis on a 1.1% agarose gel indicated that all of the DNA, including the 3.7 kb band, had been degraded, because a hazy pool of DNA appeared at the lowest portion of the gel. On the assumption that there may have been internal nicks introduced by the extraction procedure, ligase was used to try to repair them. Two ligation reaction mixtures, with appropriate buffer and ATP were incubated at 37°C for one hour. Exonuclease III was added to one of the mixtures, without changing the buffer (ligase buffer is also very low in salt), and the digestion was carried out at 37°C for one hour. Both reaction mixtures and untreated *P. parva* DNA were electrophoresed on a 1.1% agarose gel.
Mitochondrial Preparations

*P. parva* cells were harvested as described previously and pelleted in 250 ml polyethylene bottles by centrifugation for 5 minutes in a Sorval GSA rotor at 3,000 rpm. Cells were resuspended in distilled water and pelleted again. This treatment did not seem to reduce their motility.

Cells were lysed at $32^\circ$C in 750 mls of hypotonic solution (0.02 M Tris-8.0). Most of the cells were lysed within one to one and one half hours. Samples were examined periodically under the microscope. When the population was decreased to one or two intact cells per low power field, the tonicity of the solution was brought up to 0.25 M sucrose by adding 750 mls of cold 0.5 M sucrose, thereby stabilizing the unlysed cellular components. Janus green, a vital stain which turns blue when oxidized by mitochondria, was used to check to effectiveness of the lysis procedure. A large amount of blue debris indicated that many of the mitochondria were being lysed also.

The lysate was centrifuged in 250 ml bottles in the GSA rotor at 2,000 rpm for five minutes. The supernatant was carefully poured off, leaving the pellet of whole cells and free nuclei. The mitochondria, still in suspension, were pelleted by centrifugation at 6,000 rpm for 10 minutes (Cantor 1978).
The pellets were suspended in 40 ml H₂O. Non-membrane bound DNA was digested with DNase by adding the enzyme and salts (0.2 M NaCl, 5 mM MgCl₂, 10 mM sodium acetate, 3 µg/ml DNase), and incubating the solution at 4°C. The enzyme was inactivated after two hours by adding EDTA (final concentration 50 mM). The pellet was washed twice with TE buffer (10 mM Tris-8.0, 1 mM EDTA) before isolating the DNA (Brunk 1967). This treatment consistently resulted in complete disappearance of all DNA, including that in the mitochondria.
Identification of DNA in the Mitochondrial Fraction

Mitochondrial Isolation on a Sucrose Gradient

The initial mitochondrial pellet was prepared by the method described previously, except that it was not treated with DNase. The pellet was suspended in 5 ml of NET buffer.

A sucrose gradient was made in a 35 ml polyallomer tube. One and one half mls of saturated CsCl was deposited on the bottom to catch the starch pellet, and a layer of one and one half mls of 67% sucrose was added to make a cushion for the whole cells and unbroken nuclei. 17% and 55% sucrose solutions were mixed in a gradient maker to make a 21 ml gradient. The 5 ml mitochondrial suspension was layered on the gradient and there was a 3 ml space left at the top of the tube. The gradient was centrifuged for two hours at 27,000 rpm in the AH 627 Sorvall swinging bucket rotor. A yellow band of material could be seen in the middle portion of the tube.

Fractions were collected from the bottom of the tube. A peristaltic pump delivered 30 drops (one and one half mls) to each of 18 tubes in a fraction collector.

Succinic Dehydrogenase Assay

The presence of enzymatic activity was used as a
marker to identify the fractions containing mitochondria. The rate of reduction of 2,6-dichlorophenolindophenol (DCPIP) was measured at 660 nm with a Beckman Model 24 recording spectrophotometer. The amount of DCPIP was adjusted to give readings between 0.9 and 0.4 absorbance units over a three minute interval.

0.1 ml samples from each fraction were added to 1.5 ml of 0.03 M, pH 7.4 phosphate buffer, 0.5 ml 0.1 M succinate, and 0.1 ml 1 mM KCN. 40 μl of 2.5 mM DCPIP was added last. The solution was mixed in the cuvette and measured immediately. The change of absorbance per minute was calculated for each fraction from the chart paper (Rendina 1971).

DNA Extraction

The 18 fractions from the gradient were pooled to make six sequential portions. The three fractions with the greatest activity were put into one pool. The sucrose had to be removed by dialysis in order to isolate and precipitate the DNA.

Dialysis bags were fitted to pipette tips (made for the PipetteMan 5000) with half an inch of the tube removed. The bags were filled with the sucrose solutions, and the additional 5 ml space in the pipette tips allowed for a two-fold increase in volume. The solutions were dialyzed for two days against TE buffer.
The DNA was extracted as described earlier, and precipitated in 50 ml polypropylene tubes in a dry ice-ethanol bath. The tubes were centrifuged in an SW 34 rotor at 20,000 rpm for 30 minutes. The outer side of each tube was marked as it was removed from the rotor, because all but one of the DNA pellets were too small to be seen.

0.5 ml of TE buffer was used to wash out each of the DNA pellets. The large pellet which precipitated from the portion with high enzymatic activity did not completely dissolve because there was a large amount of contaminating protein. A preliminary inspection of the DNA by electrophoresis showed that there was a very large amount of RNA in the sample also. That particular sample was treated with RNase (40 μg/ml) then reprecipitated. A portion of each sample was electrophoresed and compared to the results of the enzyme assays.
Molecular Weight Estimation of the 3.7 kb Band

A log-molecular weight/migration plot of the lambda-Hind III fragments was made from a photograph, found in the results section, representing the DNA isolated from the sucrose gradient. Measurements were made from the edge of the well to each of the molecular weight standards and used to make a standard curve on semi-log graph paper. A similar measurement of the band in lane 5 was made and the size, in kilobases, was read from the graph (Rodriguez and Tait 1983).
RESULTS

Southern Blot Hybridization

Total cell DNA from P. parva and T. thermophila was transferred to nitrocellulose paper, and the 3.7 kb probe, taken directly from an agarose gel, was used for hybridization.

Most of the DNA on the gel from which the blot was taken, made a smear in lanes 1 and 2, ranging from 22 to 25 kb.

There appears to be strong hybridization with the 3.7 kb band of P. parva DNA, and a small amount of background. A very faint signal shows at 2.6 kb. There does not seem to be any hybridization with the T. thermophila DNA which was used as a control.

lane 1: Tetrahymena thermophila
lane 2: Polytomella parva
lane 3: lambda-Hind III marker
DNase Dilution Series

A portion of plasmid stock was digested with DNase at varying concentrations in order to demonstrate the change in electrophoretic mobility patterns with diminishing amounts of digestion. The photograph of a 1:2 dilution series shown below starts with the highest concentration on the left.

DNA in the first two lanes was completely digested. Lanes 3, 4, and 5 show that some of the plasmids were nicked, relaxing them to the open circular form (form II). Plasmids in lane 6 were almost entirely open circular, and lanes 7 and 8 show some of the supercoiled forms (form I).

This experiment was carried out as a model to establish a suitable method for characterizing the form of the 3.7 kb DNA in P. parva.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>form II (supercoiled)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>form I (open circular)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>form III (linear)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
*P. parva* DNA was treated in the same manner as the plasmid stock in the previous experiment, except that the eighth sample contained no DNase. Digestion of the DNA in lane one was complete, and the band did not appear to change mobilities in any of the other lanes of the gel. This pattern would be typical of a linear fraction of DNA. The faint bands that appear to run slightly ahead of the 3.7 kb fractions are degraded fragments which appear after the DNA has been stored for a long period.

In lanes seven and eight, there are distinct high molecular weight bands appearing just below the main chromosomal fraction. This DNA might also belong to the mitochondrion.
DNA Characterization with Enzymes

Exonuclease III

When *P. parva* DNA was treated with exonuclease III the entire 3.7 kb band disappeared. Prior treatment of the DNA with ligase did not effectively prevent any of the exonuclease activity.

Eco R1

Restriction of *P. parva* cellular DNA revealed a consistent banding pattern with Eco R1. It is not possible from this gel to determine whether the bands are the result of digestion of the 3.7 kb fragment, or whether they result from digestion of chromosomal sequences.

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>lane 1: λ ϕX174-Hae III digest</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lane 2: Eco R1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lane 3: Eco R1 (high conc.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lane 4: undigested</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1353+</td>
</tr>
<tr>
<td>1078+</td>
</tr>
<tr>
<td>872+</td>
</tr>
<tr>
<td>602+</td>
</tr>
</tbody>
</table>
Mitochondrial DNA Preparations

DNA that was isolated from mitochondrial membrane fragments, without prior sucrose gradient purification, appears as three distinct bands. It is not known whether the two high molecular weight bands are different conformations of the same molecule or whether the bands represent two different species of DNA.

1 2

lane 1: mitochondrial DNA preparation
lane 2: cellular DNA preparation

Seventeen fractions were taken from the sucrose gradient and analyzed for succinic dehydrogenase activity. Consecutive fractions were consolidated to form six portions in order to simplify the dialysis and DNA extractions.
Fractions four and five, taken from the sucrose gradient, show evidence that the 3.7 kb band was isolated from solutions with enzymatic activity. Fraction four originally contained the more visible pellet of nucleic acid, but there was a large amount of RNA and protein contamination. In an attempt to clean up the sample, much of the DNA may have been lost.
DISCUSSION

This was a preliminary investigation of a small DNA fragment found to exist in great abundance in the green alga, *P. parva*. The main objective of this study was to identify the source of the fragment, and to partially characterize the molecule. The 3.7 kb band as well as two larger (20 kb) bands were recovered from a mitochondrial preparation made by differential centrifugation. Although traditional methods of identifying the mtDNA were not feasible, it was shown by enzyme assays that the DNA in question associated with mitochondrial membrane fragments.

There is firm evidence that the molecule is DNA and not RNA, and that the size is approximately 3.7 kb. It would be desirable to find additional support for the supposition that the DNA is mitochondrial, however. The inability to isolate intact mitochondria as a source of mtDNA also hindered our ability to characterize the fragment. The molecule appears to be linear with unprotected ends, and its sequence seems to be present only in the 3.7 kb DNA, but these results need to be confirmed by additional experiments.

The problems of acquiring adequate mitochondrial preparations have to be resolved before more progress can be made on the project. There were two other procedures that had potential and were not tried because of
time constraints and lack of equipment. Simpson has developed an excellent method for isolating trypanosomal kinesomes by forcing hypotonically swollen organisms through a small orifice under a constant pressure controlled by nitrogen gas (personal communication, 1985). A French pressure cell also might have been used to break the cells open without ripping the mitochondrial membranes. If a sufficiently pure mitochondrial preparation were to be obtained, then the mtDNA could be identified and extracted in the usual manner.

Another approach could be used to circumvent the mitochondrial isolation difficulties by using two steps. First, the location of the mtDNA could be demonstrated by "in situ" hybridization. Biotinylated probes have been used recently to identify homologous sequences on paraffin mounted thin sections. Electron micrographs would show the mitochondrial membranes as well as the labeled DNA (BRL technical representative, personal communication 1985).

The second problem, that of obtaining a pure sample of the fragment, could be eliminated by isolating the 3.7 kb DNA in a CsCl-EtBr gradient preparation of total P. parva DNA. A clean supply of DNA would be more easily characterized.

Ideally, the shape of the molecule should be determined by electron microscopy. A similar organism, Chlamydomonas reinhardi, was originally found to have linear
mtDNA. When the molecules were inspected by electron microscopy, a very small portion (<1%) were found to be circular. This information also contributed to the understanding of its replication mechanism (Ryan et al. 1978). The apparent linear conformation of *P. parva*'s mitochondrial fragment should be confirmed by a similar method or by restriction site mapping. An attempt to construct a restriction map was made by digesting total cellular DNA, but was not successful due to the presence of many incompletely digested fragments.

Because of its greater sensitivity, it would have been preferable to use a radioactive probe rather than the enzymatic system, to try to detect the presence of the 3.7 kb sequence within the larger DNA. According to Murasugi and Wallace (1984), there is a 1,000-fold difference in sensitivity between the two methods. There was enough material to make a satisfactory probe by isolating the fragments from a low melting point agarose gel, but purification of the biotinylated probes was rather tedious. I was not able to reuse the leftover hybridization solution after it had been stored at 4°C for two months. Probe stability was supposed to have been the major advantage to the system.

A more thorough characterization of the ends of the 3.7 kb DNA molecules would include an attempt at end-labeling with terminal transferase. This could have been done with biotinylated dUTPs as well. Like exonuclease III,
the enzyme requires free 3'OH groups. The 5' ends could have been tested by incubating the fragments with polynucleotide kinase and 32\gammaATPs. When a similar linear mtDNA plasmid from maize was tested, the fragments appeared to be sensitive to exonuclease III and terminal transferase, but insensitive to kinase. It was later determined that proteins were covalently linked to the 5' termini. It was hypothesized that the DNA-protein complexes were involved in priming the replication of the linear DNAs (Sederoff 1984). This aspect of the P. parva molecule should be studied further.

Chlamydomonas reinhardi is the only green alga with mtDNA publications to its credit. The organism has a singular mtDNA molecule that is surprisingly small, about 16 kb. (Most plant and algal mtDNAs are at least ten times that size.) The fact that the molecules are nearly all linear was another unexpected discovery (Ryan et al. 1978). The major mtDNA molecule of P. parva is not much larger than C. reinhardi's at 20 kb. It would be very interesting to find out if it is linear also.

Small mitochondrial DNA elements have been found in yeast, fungi, and higher plants. The kinetoplast of trypanosomes contain a huge network of large and small circular molecules called minicircles and maxicircles. Some plant mtDNAs also consist of a heterogeneous mix of sizes.

Bean mtDNA isolated from the supercoil regions of
CsCl-EtBr gradients displayed an oligomeric series of 30 bands. Corn mtDNA electrophoretic patterns were less complex, having two dominant species of 1.5 and 1.8 kb. The mtDNA from tobacco had 10-12 bands ranging from 10 to 29 kb. Hybridization studies indicated that there was homology among the different size classes in each of these organisms suggesting that the genomes were not as complex as their mtDNA electrophoretic patterns had first indicated (Dale 1982).

Cultures of *Podospera anserina* were harvested at different phases during its life span. Amplified mtDNA sequences were found to increase in quantity with senescence. Unlike *P. parva*, the sequence was able to hybridize with the large juvenile mtDNA. *Neurospora crassa* and *N. intermedia* also have small circular mtDNAs, about 4 kb, but they do not hybridize with the large mitochondrial loops, and there is no significant homology between the small circles in the two different species (Sederoff 1984). It appears that some, but not all, small mtDNA fragments are amplified duplications of portions of the primary mtDNA. Before assuming this is not the case for *P. parva*, it would be wise to repeat the hybridization experiment with radioactive probes to prove that the 3.7 kb segment was not present in the major mtDNA band.

The purpose of these extra mtDNA elements is still unknown. Some of the sequences contain open reading frames, such as the linear maize plasmids (Sederoff 1984),
but many of them do not. Evolutionary trees have been based on mtDNA differences, and recent evidence indicates that mutations are fixed 5 to 10 times more quickly in the mitochondrion than in the nucleus (Wilson seminar 1985). Unicellular organisms have demonstrated some of the extremes of mtDNA design. We do not know whether the most primitive organisms had only one size category of mtDNA or several. It is fairly obvious now that animals tend to have one small mtDNA loop, and plants most often have a loop that is ten-fold larger than the animal mtDNA, as well as a mixture of smaller molecules. Many of the green algae have both "plant-like" and "animal-like" features and cannot be unequivocally assigned to one kingdom or the other. Now we have two organisms to compare. C. reinhardi and P. parva both appear to have small, "animal-like" primary mtDNA strands. With the additional mitochondrial plasmids, P. parva is more similar to plants than C. reinhardi.

Both algae have been classified in the order of Volvocales and the class Chlorophyceae (W. D. Stewart 1974). Protozoologists put them in the class Phytomastigophorea. The most obvious distinction between the two organisms is that C. reinhardi has chlorophyll and a cell wall that contains cellulose, whereas P. parva has no chlorophyll and lacks a cell wall.

There are many "colorless" algae that are distinguished from the photosynthetic forms solely by the absence
of chlorophyll. It is not entirely clear how the loss of the photosynthetic apparatus occurs, but for some cells, the accident can be survived. The colorless counterpart to Chlamydomonas is Polytoma (the organism for which Polytomella was named), and the colored species corresponding to Polytomella is Dunaliella (W. D. Stewart 1974). It would be very interesting to see if Dunaliella contains the same 3.7 kb fragment as Polytomella.
REFERENCES


