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# A MINICIRCULAR COMPONENT OF ACETABULARIA ACETABULUM CHLOROPLAST DNA REPLICATING BY THE ROLLING CIRCLE.

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## SUMMARY

Data are presented which show that a population of covalently closed minicircles, 0.1 to 1.5 μm long, represent, on a DNA length basis, approximately 0.5% of the total chloroplast genome of Acetabularia acetabulum.

With a frequency of about 50% minicircles appear to undergo DNA replication following the rolling circle model.

The significance of these findings is discussed.

## INTRODUCTION

In the course of an extensive ultrastructural characterization (16, 17) of the chloroplast genome of Acetabularia (A.) acetabulum, it occurred to observe that about 0.5% (on a DNA length basis) of the total chloroplast genome is represented by covalently closed minicircles of heterogeneous contour lengths ranging from 0.1 to 1.5 μm. In addition, with a frequency of about 50%, these minicircles appeared to undergo active DNA replication following the rolling circle model (8).

The purpose of this report is to both illustrate and comment the results of electron microscope studies on the above material.

## MATERIALS AND METHODS

Methods for isolation and purification of sterile chloroplast pellets and uncontaminated chloroplast DNA have been already described (16, 17).

To summarize: Axenically grown (2) Acetabularia cells at stage 4 (2) have been chosen.

At this stage of growth the whole nucleus (a possible source of contaminating DNA) resides in one of the branches of the rhizoid (2, 12) and can be easly snipped off. This has also the advantage of eliminating the major, or unique, source of contamination from bacterial DNA (2, 9).

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Thus the axenic (sterile) Acetabularia cells were enucleated and subjected to the procedures for either purification of chloroplast DNA (17) or electron microscope observation of osmotically shocked chloroplasts of individual Acetabularia cells (16).

The above conditions guarantee 100% pure chloroplast DNA preparations (16, 17).

Electron microscopy and length measurements of DNA molecules have been previously described (16, 17, 18) and are detailed in the legends of figures.

## RESULTS

The above procedures allow isolation of Acetabularia chloroplast DNA under a single homogeneous peak, at a buoyant density of about 1.703 g/ml (fig. 1A), which does not change after repeated rebanding in CsCl gradients (fig. 1B).

When examined by electron microscopy, the very central fraction of the peak appears to be constituted primarily of either free molecules (40%) or supramolecular complexes (55%) in form of displays (fig. 2a,c).

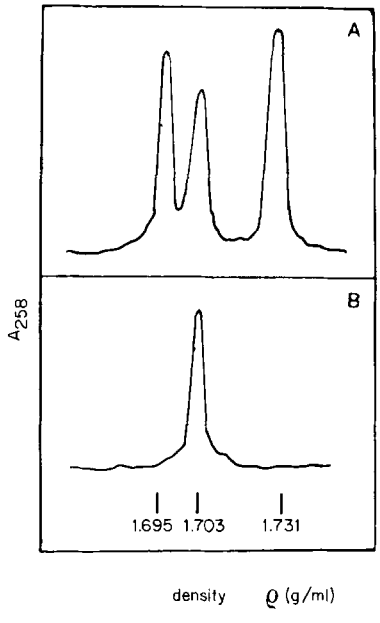


Fig. 1. Density determination and CsCl purification of sepharose-sieved chloroplast DNA (17): (A) Chloroplast DNA has a buoyant density of 1.703 g/ml relative to Clostridium perfringens (1.695 g/ml) and Streptomyces coelicolor (1.731 g/ml) DNA. (B) CsCl rebanding of the 1.703 material gives a uniform, homogeneous peak indicative of absence of contaminating DNA.

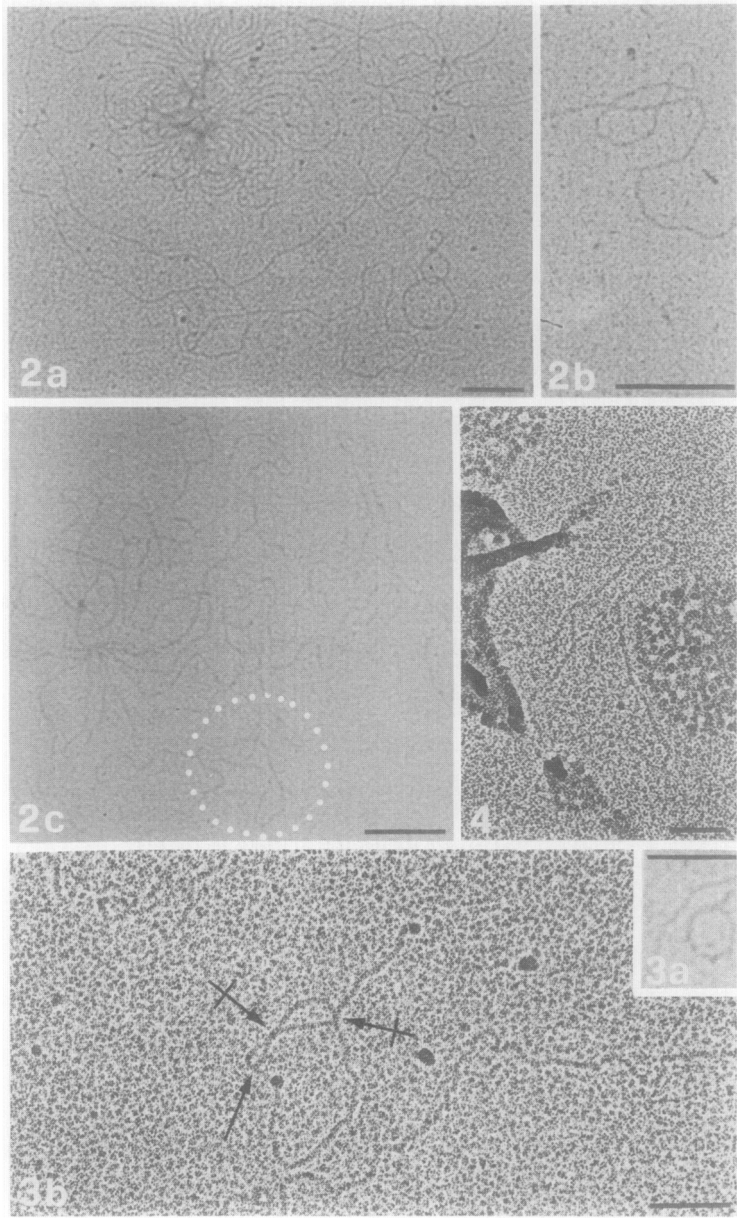


Fig. 2.: a 0.5 μm open minicircle interlocked with a broken 0.4 μm minicircle, in close proximity with peripheral strands of a 100 μm DNA display partially included in the picture (a); a 0.5 μm overtwisted (b) and a 0.6 μm open (c, encircled area) minicircle interlocked with peripheral strands of displays.
Microdroplet diffusion (5) spreading (a); formamide (7) spreading (b, c); uranyl-formol (18) staining (a, b, c). Bar equals 0.2 μm.

Fig. 3. Rolling circle intermediates with: (a) the smallest (circle 0.17 μm, tail 0.01 μm) and (b) an intermediate (circle 0.57 μm, tail 0.26 μm), replicating tail size.

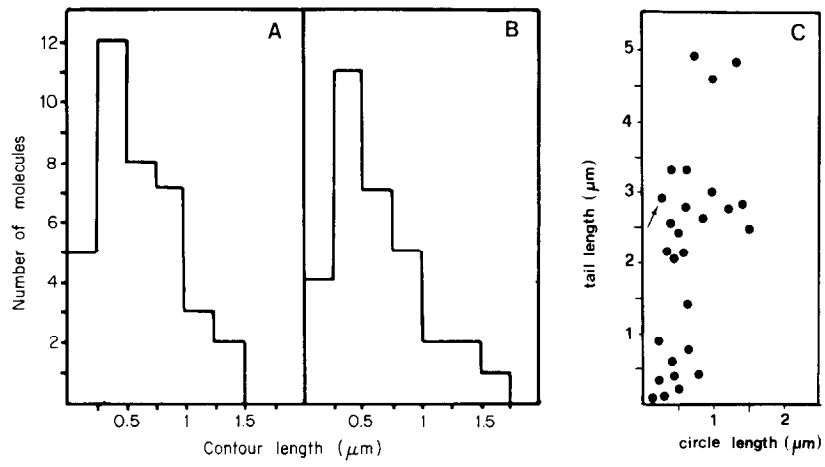


Fig. 5. Size distribution of unreplicating (A) and replicating (B) minicircles. Both diagrams show peaks at 0.37 μm and identical length distribution classes. Mean: 0.6 μm ± 0.7 μm. (C) Plot of tail versus circle length in replicating minicircles. Notice the largest replication extents in the 0.2 μm class of minicircles.
Length measurements according to Lang et al. (15).

However a very minor fraction (0.5% on a DNA length basis) is represented by covalently closed minicircular DNA molecules either open (fig. 2a) or overtwisted (fig. 2b).

On many occasions minicircles lie in very close proximity (fig. 2a), or inter-locked with peripheral strands of typical chloroplast DNA displays (fig. 2b, c). In a proportion of about 50% (fig. 5A, B) minicircles appear to be undergoing active DNA replication by the rolling circle mechanism (fig. 3a, b).

Size distribution diagrams of either replicating (fig. 5B) or unreplicated (fig. 5A) minicircles show that both belong to a virtually identical population with a modal peak around 0.37 μm and 0.6 μm mean contour length (fig. 5A, B).

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The starting point (arrow) of replicating tail and its intertwining with the circle (crossed arrows) are clearly evident in fig. 3b.
Formamide (7) spreading; uranyl-formol (18) staining (a): Pd shadowing (16; b). Bar equals 0.1 μm.

Fig. 4. A 1.2 μm, partially overtwisted, rolling circle with a 0.1 μm tail in close connection with typical remnants of the chloroplast membrane which apparently shape the contour of the molecule.
Osmotically shocked and Pd shadowed chloroplasts (16). Bar equals 0.1 μm.

The extent of replication ranges from 0.1 (fig. 3a and 5C) to about 11 (fig. 5C, arrow) times the minicircle length, having the smallest circles the largest replicating tails.

Electron microscopy of osmotically shocked chloroplasts of individual Acetabularia cells shows that both unreplicated and replicating (fig. 4) minicircles are very often in close connection to the chloroplast membrane. Statistical data on this material have been incorporated in the above histograms.

## DISCUSSION

Cytoplasmic, minicircular DNA's are now known to be widely distributed in both animal and vegetal kingdoms (e.g. see 4, 5, 10, 11, 20).

However, in the context of present discussion, minicircular DNA, associated with the chloroplasts of the closely related A. cliftonii species (10), merits particular attention.

Both A. cliftonii (10) and A. acetabulum (this work) minicircular DNA's appear, in all likelihood, to be located within the chloroplastic membrane, as suggested in both cases by electron microscopy of osmotically shocked chloroplasts.

However, minicircular DNA individuated by us in A. acetabulum appears to differ from that of A. cliftonii in many respects:

1) It has much smaller size: $\sim 0.6$ μm mean contour length ($\sim 1.2 \times 10^6$ daltons) as opposed to $\sim 4.2$ μm ($\sim 8.7 \times 10^6$ daltons) in A. cliftonii (10);
2) It appears to be very actively replicating under conditions of normal vegetative growth of the plant, while no replicative intermediates were reported in A. cliftonii (10);
3) It appears to be located under the very central portion of the 1.703 CsCl peak and, as shown electron microscopy, intimately connected with the bulk chloroplast DNA; A. cliftonii minicircular DNA is reported to have 1.712 buoyant density (10);
4) It is not separable from the 1.703 chloroplast DNA fraction in ethidium bromide-CsCl gradients (Bonotto, unpublished).

All these facts lead to the conclusion that minicircular DNA, altough very small, is an integral part of the chloroplast genome of A. acetabulum, perhaps very different from that of A. cliftonii.

Its very low amount and intimate connection with the bulk 1.703 chloroplast DNA sufficiently accounts, in our opinion, for its lack of resolution in ethidium bromide-CsCl gradients. Experimental conditions are now being sought to increase its content and/or release it from tight connection with the rest of the chloroplast genome.

Its replication mechanism is not unusual for a DNA of chloroplastic origin. Rolling circle intermediates have already been reported in chloroplast DNA of higher plants as the completion step of a complex model of replication, sequentially involving both the Cairns and the rolling circle mechanism (14).

Cairns intermediates have not been found in our material as yet.

Being the rolling circle a gene amplification (13) mechanism and knowing that A. acetabulum chloroplasts are endowed with both 16 and 23S rRNA (3) and actively engaged in protein and RNA synthesis (6), it is tempting to speculate, that DNA minicircles could carry cistrons for both t-and r-RNA's.

As evidenced in higher plants chloroplasts (1) 16 and 23S rRNA's may be calculated to be 0.45 and 0.91 μm long respectively.

Wether both are synthesized as a single precursor unit of a calculable 1.3 μm length, as in Chlamydomonas (19), or individually it is not known.

In either of the two eveniences the role of rRNA carrying genes can be ascribed only to minicircles of discrete length classes, the others representing either n-meres of the previous ones or even enrelated genes.

Electron microscope techniques of partial melting (14) and RNA-DNA hybridization (1) are now being employed to solve these problems.

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