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Tandemly repeated nonribosomal DNA sequences in the chloroplast genome of an *Acetabularia mediterranea* strain

(Southern blot/molecular cloning/Dasycladaceae/evolution)

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ABSTRACT **A purified chloroplast fraction was prepared from caps of the giant unicellular green alga *Acetabularia mediterranea* (strain 17). High molecular weight DNA obtained from these chloroplasts contains at least five copies of a 10-kilobase-pair (kbp) sequence tandemly arranged. This unique sequence is present in DNA from chloroplasts of all stages of the life cycle examined. A chloroplast rDNA clone from mustard hybridized with some restriction fragments from *Acetabularia* chloroplast DNA but not with the repeated sequence. An 8-kbp *Eco*RI–*Pst* I fragment of the repeated sequence was cloned into pBR322 and used as a hybridization probe. No homology was found between the cloned 8-kbp sequence and chloroplast DNA from related species *Acetabularia crenulata* or chloroplast DNA from spinach.**

Chloroplast genomes are usually considered to have a conservative structure and to be circular with a contour length of 37–46 μm in higher plants. A larger size range of 25–60 μm occurs for the circular DNA of some green algae. Despite a sometimes long evolutionary separation, all chloroplast genomes so far examined have shown significant similarities. Chloroplast genomes of widely seperated plants, such as the liverwort *Marchantia polymorpha* (1), the fern *Osmunda cinnamomea* (2), the green alga *Chlamydomonas reinhardii* (3), and spinach (4), have two copies of a sequence that contains ribosomal RNA genes arranged in an inverted orientation with a small and a large single-copy DNA region between them. Arrangements with no inverted repeat have been found in tribe Vivieae of Leguminoceae where only one ribosomal gene set is found (5). In the alga *Euglena gracilis*, three tandem repeats containing ribosomal RNA genes are found (6). There has been no indication that any other large stretches of the chloroplast genome are reiterated. Although a lot is now known about the physical structure of chloroplast genomes, this should not obscure the fact that only a low number of plant taxa, most of them closely related, have been examined so far.

One of the most ancient families of green algae, Dacycladaceae, which is known by numerous fossil species, has a number of present day representatives of which *Acetabularia* is the best known. Early studies with *Acetabularia* have shown that DNA from chloroplast fractions contains long lengths of DNA when viewed in the electron microscope (7). The kinetic complexity of this DNA resembles that of *Escherichia coli* rather than that of *Chlamydomonas* (8). With this information we have started a study to further characterize the chloroplast genome of *Acetabularia*.

In this paper we describe experiments that reveal that the chloroplast genome in a strain of *Acetabularia mediterranea* contains tandemly repeated sequences that are stable during the life cycle and that are not homologous to heterologous probes for ribosomal RNA genes.

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MATERIALS AND METHODS

Preparation of Chloroplasts. *A. mediterranea* was grown in Müller's medium as described (for references, see ref. 9). Cells of three different stages, 1 cm long, 3.5 cm long (i.e., just prior to cap formation), and fully developed caps (9) were studied.

Caps from *A. mediterranea* cells were harvested prior to the formation of secondary nuclei. Five-thousand caps (≈100 g) were homogenized in a blender fitted with razor blades on a vertical shaft in 1 liter of ice-cold buffer A containing 0.6 M sorbitol, 0.1 M 2-{[tris(hydroxymethyl) methyl]amino}ethanesulfonic acid (Tes; pH 7.8), 0.005 M disodium EDTA, and 0.002 M 2,3-dihydroxy-1,4-dithiobutane.

After five 3-sec bursts in the blender, the homogenate was filtered through two layers of Miracloth (Calbiochem) and 1 layer of 10-μm nylon cloth. Chloroplasts were pelleted by centrifugation at 3500 rpm in a GSA rotor (Sorvall) for 4 min at 4°C. The crude chloroplast pellets were suspended in 400 ml of buffer B containing 0.6 M sorbitol, 0.01 M Tes (pH 7.8), 0.005 M EDTA, and 0.002 M 2,3-dihydroxy-1, 4-dithiobutane. After a minimum of 1 hr at 0°C to reduce aggregation, the chloroplast suspension was filtered slowly through 5-μm Nuclepore polycarbonate membranes with use of a syringe. Each 50-mm filter could filter 40–50 ml of suspension before centrifugation for 5 min at 4000 rpm in an SS-34 rotor (Sorvall). Chloroplasts were resuspended in buffer B, recentrifuged, and resuspended in a total of 20 ml of buffer B. Five ml of chloroplasts were then layered over each of four 20-ml linear 10–60% (vol/vol) Percoll (Pharmacia) gradients in buffer B in Corex glass tubes (Sorvall). After centrifugation at 5000 rpm for 30 min in a HB4 rotor (Sorvall), the main lower chloroplast band at ≈50% Percoll, which contained intact chloroplasts, was collected with a wide-bore Pasteur pipette. To remove the Percoll, chloroplasts were diluted 1:4 with buffer B and pelletted by centrifugation at 5000 rpm for 10 min in a HB4 rotor. This washing step was repeated once. The sample was checked for purity by electron microscopy.

Preparation of DNA from Chloroplasts (ctDNA). Chloroplast pellets from 100 g of caps were suspended in 10 ml of 0.05 M Tris, pH 8.0/0.12 M EDTA/500 μg of proteinase K (Merck, 0.02 Anson units per mg) per ml and held on ice for 30 min. Two ml of 20% (wt/vol) sodium *N*-laurylsarcosinate was added dropwise with gentle mixing, and lysed chloroplasts were incubated at 0°C for 30 min. Solid CsCl at 3 g per 10 ml of lysate was then added and dissolved by rolling the tube on ice. When the CsCl was dissolved, the lysate was centrifuged at 10,000 rpm in an SS-34 rotor, and the pellet, which contained starch and insoluble protein, was discarded. To the supernatant was added additional CsCl to

Abbreviations: ctDNA, chloroplast DNA; kbp, kilobase pairs.

give a final concentration of 1 g·ml^{-1} of lysate, and the tube then was warmed to 20°C to allow the CsCl to dissolve. Ethidium bromide (10 mg·ml^{-1} in water) was added at 1 ml per 10 ml of lysate, and the density was adjusted to 1.58 g·ml^{-1}. After centrifugation at 40,000 rpm for 40 hr in a Ti 50 rotor (Beckman), the DNA bands were pooled and rerun. Then, the final DNA fraction was extracted with butanol saturated with CsCl and 0.05 M Tris/0.01 M EDTA, pH 8, to remove ethidium bromide and dialyzed against 0.01 M Tris·HCl, pH 8.0/0.0001 M EDTA for 2 days with frequent changes of buffer.

Cloning of Repeated Sequence. *A. mediterranea* strain 17 DNA (20 μg) was digested to completion with *Eco*RI and *Pst* I and fractionated on a 0.7% low melting point agarose (Bethesda Research Laboratories) gel. The major repeat band was excised, and the DNA was recovered as described by Bedbrook *et al.* (10). pBR322 was digested to completion with *Eco*RI and *Pst* I, and the large plasmid fragment was prepared by fractionation on a 1.0% low-melting-point gel.

Ligation of 0.5 μg of pBR322 fragment and 0.5 μg of *Eco*RI–*Pst* I sequence was performed at 12°C for 6 hr with 1 unit of T4 ligase (Bethesda Research Laboratories) in 20 μl of the ligation buffer recommended by the supplier. The ligation mix was used directly to transform 0.2 ml of competent *E. coli* C 600 cells by the procedure of Mandel and Higa (11). Transformed cells were screened for tetracycline resistance and ampicillin sensitivity, and potential clones were further examined in minipreparations (12).

Labeling of DNA. Nick-translation with [α-^{32}P]dCTP (800 Ci·mmol^{-1}, Amersham–Buchler; 1 Ci = 37 GBq) was performed according to Rigby *et al.* (13). End-labeling of DNA with recessed 3′ termini produced by *Eco*RI was achieved by using [α-^{32}P]dATP (3000 Ci·mmol^{-1}, Amersham–Buchler) and the Klenow fragment of *E. coli* DNA polymerase (Bethesda Research Laboratories) in nick-translation buffer. End-labeling of DNA with protruding 3′ termini (*Bgl* I, *Pst* I) was achieved by using either cordycepin [α-^{32}P]triphosphate or [α-^{32}P]dATP (3000 Ci·mmol^{-1}, Amersham–Buchler) and terminal transferase (Bethesda Research Laboratories) by using the protocol suggested by Bethesda Research Laboratories. All labeled DNA was extracted with phenol and precipitated twice with one vol of 7.5 M ammonium acetate and two vol of ethanol at −80°C for 1 hr before use.

Gel Electrophoresis, Southern Blots, and Filter Hybridization. Electrophoresis on agarose gels was performed in a horizontal (submarine) gel, 0.5 cm thick and 25 cm long, at 25 V for 0.6% gels and at 50 V for 1–1.5% gels in buffer containing 0.04 M Tris acetate (pH 8.0), 0.02 M Na acetate, and 0.002 M EDTA.

Molecular size standards for 0.6% agarose gels were prepared by digestion of phage λ DNA with *Hin*dIII, *Kpn* I, *Eco*RI, *Pvu* I, and *Sal* I. When mixed, these single-enzyme digests, together with undigested phage λ DNA, provide molecular size standards ranging from 2 to 48.5 kilobase pairs (kbp) (14). Transfer of DNA to nitrocellulose filters (Schleicher & Schüll BA 85) was performed by the method of Southern (15). Filters were prehybridized in 0.9 M NaCl/0.09 M sodium citrate, pH 7.0/0.01 M EDTA/5× Denhardt's solution (16)/10 μg of boiled calf thymus DNA per ml at 65°C for 2 hr in heat-sealable plastic bags. Hybridizations were performed at 60–65°C in prehybridization buffer containing 0.2% sodium dodecyl sulfate, calf thymus DNA at 100 μg·ml^{-1}, and 0.5–10 × 10^6 cpm of ^{32}P-labeled probe, which was then boiled for 10 min prior to its addition to the bag containing the filter. After incubation for 24 hr at 60–65°C depending on the experiment, filters were washed in 0.3 M NaCl/0.03 M sodium citrate, pH 7.0/0.2% sodium dodecyl sulfate at 60–65°C for 1 hr and then in 0.015 M NaCl/0.0015 M sodium citrate, pH 7.0, for a stringent wash or in 0.15 M NaCl/0.015 M sodium citrate, pH 7.0, for a nonstringent wash for another 2 hr with two changes of the solvent. In most experiments phage λ DNA-derived molecular size standards were visualized by including an appropriate amount of nick-translated phage λ DNA with the hybridization probe. Autoradiography at −80°C was performed by using Agfa Curex x-ray film with DuPont Hi-Plus intensifying screens.

RESULTS

Preparation of ctDNA. A major limitation in working with ctDNA in *Acetabularia* has been the problem of obtaining sufficient quantities for analysis. A recent study of *Acetabularia* chloroplasts in which fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) was used to stain DNA has demonstrated that virtually all chloroplasts in caps contain DNA (17). We took advantage of this observation and developed an improved method for the preparation of *Acetabularia* ctDNA.

This method uses a chloroplast purification scheme that works well with higher plant chloroplasts. A filtration step with a 5-μm membrane has been used because it has been found to be effective in disrupting cytoplasts that are formed when *Acetabularia* cells are chopped (18). The extraction buffer has a high buffering capacity because vacuoles in the *Acetabularia* cells contribute a large amount of acid to the medium. Another improvement in our procedure is the use of a Percoll density gradient to separate intact chloroplasts from broken chloroplasts and other contaminations (19). In the resulting chloroplast fraction, no bacteria and less than one mitochondrion per five chloroplasts were detected by electron microscopy. DNA from intact chloroplasts collected from Percoll gradients gave a homogenous profile on neutral CsCl gradients with a density of 1.704 g·cm^{-3} (data not shown). This value agrees with values reported previously (20, 21). We also analyzed DNA from a crude mitochondrial preparation and from gametes and found densities of 1.716 and 1.695 g·cm^{-3}, respectively, which also agrees well with values of 1.715 and 1.696 g·cm^{-3} reported for mitochondrial and nuclear DNA (20, 21). In DNA preparations from Percoll gradient-purified chloroplasts, there was no indication for the presence of mitochondrial DNA. Since chloroplasts were prepared from caps, contamination with nuclei also can be excluded. To prepare DNA with a high molecular weight, we found it necessary to lyse chloroplasts in buffer containing proteinase K and a high EDTA concentration. With this extraction procedure, *A. mediterranea* ctDNA with an average molecular weight of ≈140 kbp was obtained as judged on the basis of agarose gel electrophoresis. The yield was typically 100 μg of DNA from 300 g of caps (fresh weight).

Restriction Enzyme Analysis of ctDNA. Digestion of *Acetabularia* ctDNA with the restriction enzymes *Sal* I, *Bam*HI, *Bal* I, and *Kpn* I, which give simple patterns with ctDNA of higher plants, resulted in rather complex patterns (Fig. 1, tracks b, c, d, and f). A striking feature of digests with *Kpn* I, *Eco*RI, and *Bgl* I is an intense band corresponding to a size of 10 kbp (Fig. 1, tracks f, g, and h). This band is also found in DNA digested with *Bgl* I from young cells 1 cm in length and also in cells prior to cap formation (Fig. 1, tracks i and j), which suggests that the *Bgl* I-generated band is a stable feature of the DNA and not a stage-specific DNA amplification.

The band of *Bgl* I fragments in ctDNA from caps was analyzed by sequential digestion of DNA prepared on low-melting-point gels. The *Bgl* I repeated sequence (Fig. 2, track a) was digested with *Nru* I, *Eco*RI, and *Hae* II (Fig. 2, tracks b, c, and d). Bands produced by the second digestion add up to the molecular weight of the fragments digested, which indicates that the *Bgl* I repeated sequence represents

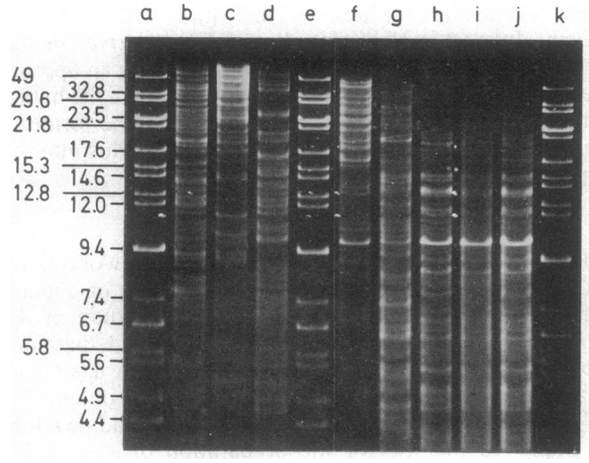

FIG. 1. Restriction pattern of ctDNA with different endonucleases. Cleaved DNA was size-separated on a 0.6% agarose gel, and bands were visualized by means of ethidium bromide fluorescence. Tracks: a, e, and k, identical size markers, which have the molecular sizes (shown in kbp) indicated next to track a; b–d and f–h, ctDNA from caps digested with *Sal* I, *Bam*HI, *Bal* I, *Kpn* I, *Eco*RI, and *Bgl* I, respectively; i and j, *Bgl* I digests of ctDNA from 1-cm-long cells and cells just prior to cap formation, respectively.

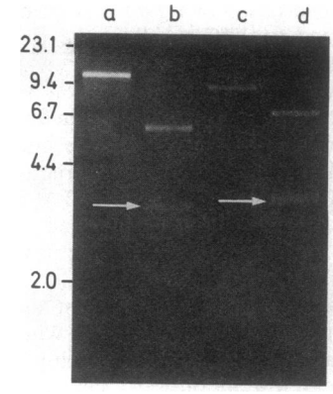

FIG. 2. Restriction analysis of the *Bgl* I-generated repeated sequence. The *Bgl* I repeated sequence was prepared by size-fractionation on a 0.6% low-melting-point agarose gel. The band containing the 10-kbp repeated sequence was cut from the gel and heated to melt the agarose, and digests with restriction endonucleases were performed. DNA molecular size markers are indicated by the numbers next to track a. Tracks: a, *Bgl* I-gene-rated repeating sequence excised from a low-melting-point gel; b–d, digests of the *Bgl* I sequence with *Nru* I, *Eco*RI, and *Hae* II, respectively. The position of two small bands not clearly reproduced in the photograph are shown with arrows.

a homogenous population of molecules. Further analysis of the *Bgl* I repeated sequence revealed that the *Eco*RI and *Kpn* I 10-kbp fragment resulted from one larger sequence (see also Fig. 5). The arrangement of restriction sites for some enzymes within the 10-kbp *Bgl* I repeated sequence is shown in Fig. 3. *Bam*HI and *Sal* I did not cut the repeated sequence.

Cloning of the Repeated Sequence. To facilitate analysis of the distribution of the repeated sequence in the chloroplast genome of *Acetabularia*, the 8-kbp *Eco*RI–*Pst* I sequence within the *Bgl* I repeat was cloned in pBR322. The DNA was inserted between the unique *Eco*RI and *Pst* I sites of pBR322, thus resulting in a directional insertion (Fig. 3*B*). One clone, pAmc3, which showed the required insert size and *Hae* II site, was chosen for further restriction analysis. The *Eco*RI–*Pst* I insert was end-labeled with dideoxy ATP by using terminal transferase. Chloroplast DNA was digested with *Pst* I and *Eco*RI, and the 8-kbp *Eco*RI–*Pst* I sequence was prepared from low-melting-point gels as described and end-labeled in the same way as the cloned fragment. Restriction analysis of these end-labeled fragments confirmed, within the limits of the analysis, that the clone was the same as the uncloned *Eco*RI–*Pst* I repeated sequence.

Disposition of the Repeated Sequence. The cloned *Eco*RI–*Pst* I sequence prepared from pAmc3, was nick-translated and hybridized with filter-bound restriction digests of total ctDNA. With enzymes *Bgl* I and *Eco*RI, 10-kbp portions of the repeated sequence gave a strong signal (Fig. 4, tracks b and c). *Sal* I (Fig. 4, track a) showed a strong signal corresponding with a size greater than 50 kbp. When the single-enzyme digests were digested additionally with *Pst* I and *Eco*RI (Fig. 4, tracks d, e, and f), virtually all of the signal was found in the band containing the *Eco*RI–*Pst* I sequence. The minor signal of 17.5 kbp, found when the cloned *Eco*RI–*Pst* I sequence was hybridized to total DNA digested with *Bgl* I (Fig. 4, track b), might be a restriction fragment directly adjacent to the *Bgl* I repeat. This fragment probably contains the *Eco*RI–*Pst* I sequence because triple digests with *Bgl* I, *Eco*RI, and *Pst* I showed virtually all of the hybridization signal in the band corresponding to the *Eco*RI–*Pst* I sequence (Fig. 4, track e).

Five restriction enzymes of 25 tested showed a repeated sequence of 10 kbp. The cloned *Eco*RI–*Pst* I sequence hybridized to a 10-kbp band with all five enzymes (Fig. 5). The production of identically sized fragments with a number of enzymes suggests that the repeated sequence is arranged in tandem, since enzymes that cut once in a tandem repeat would produce fragments with the unit size of the repeated sequence. Enzymes that cut more than once should show bands smaller in size than the whole repeat. *Pst* I, *Nru* I, and *Hpa* I cut the repeated sequence more than once and, thus, produced small molecular weight bands (Fig. 5, tracks g, h, and i).

If the repeated sequence were arranged in tandem, a partial digestion of high molecular weight ctDNA with *Bgl* I should show multimers of the basic 10-kbp sequence. In fact, size fractionation on an agarose gel of partial *Bgl* I digestion

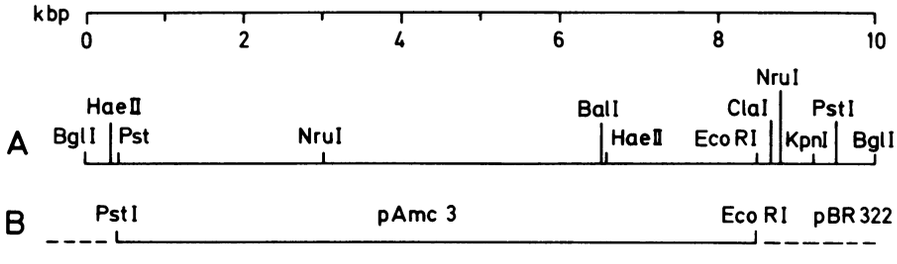

FIG. 3. Restriction maps of the *Bgl* I-generated repeated sequence and the clone pAmc3, which contains the *Eco*RI–*Pst* I sequence cloned into *Pst* I and *Eco*RI sites of pBr322. The cleave sites for only some infrequently cutting enzymes are shown. (*A*) *Bgl* I repeated sequence. (*B*) pBR322 construct containing the 8-kbp *Eco*RI–*Pst* I sequence.

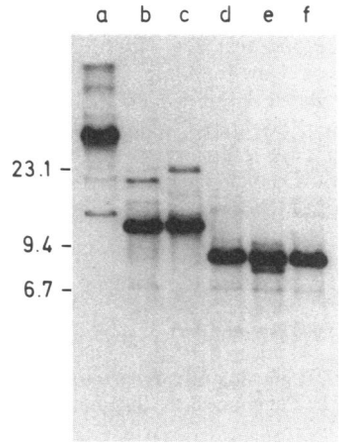

FIG. 4. Autoradiograph showing hybridization of the cloned *Eco*RI–*Pst* I sequence to Southern blots of ctDNA digested with restriction enzymes. The position of some molecular size markers (shown in kbp) are indicated by numbers next to track a. Tracks: a–c are digests with enzymes *Sal* I, *Bgl* I, and *Eco*RI, respectively; d, double digest with *Pst* I and *Eco*RI; e and f, triple digests in which *Bgl* I and *Sal* I single-enzyme digests are additionally digested with *Pst* I and *Eco*RI, respectively.

products, Southern blotting, and hybridization with the cloned *Eco*RI–*Pst* I sequence revealed bands that represent multimers up to pentamers (Fig. 6, tracks b–i). This suggests that the repeated sequence is present at least once in the genome as a tandem repeat of five or more repeated units.

Nature of the Repeated Sequence. The only large sequence reported in the majority of chloroplast genomes studied to date is a 20- to 28-kbp inverted repeat that contains ribosomal RNA genes (22). In *Euglena* three copies of a 5.6-kbp DNA segment that contain ribosomal genes are tandemly arranged (6).

To examine if the *A. mediterranea* repeated sequence

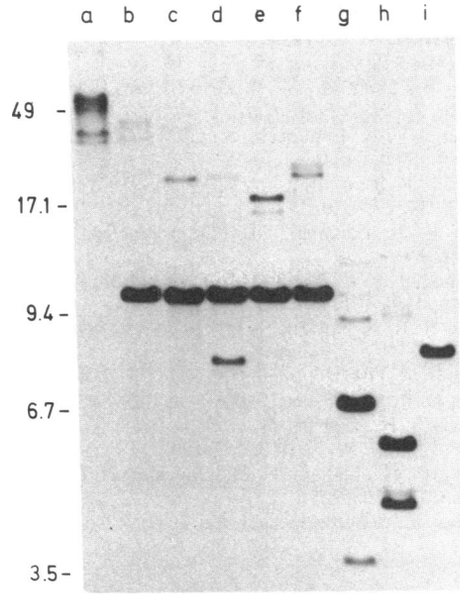

FIG. 5. Autoradiograph showing hybridization of the cloned *Eco*RI–*Pst* I sequence to a Southern blot of ctDNA digested with restriction enzymes. The position of molecular size markers (shown in kbp) are indicated by numbers next to track a. Tracks: a, digest with *Pvu* I; b–f, digests with *Kpn* I, *Cla* I, *Bal* I, *Bgl* I, and *Eco*RI, respectively, which produce a 10-kbp band of restriction fragments; g–i, digests with enzymes *Hae* II, *Nru* I, and *Hpa* I, respectively, which result in smaller repeated sequences.

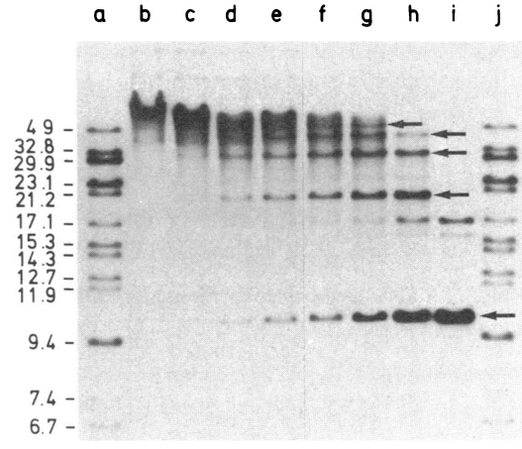

FIG. 6. Autoradiograph showing the hybridization of the cloned *Eco*RI–*Pst* I sequence to a Southern blot of a time-course digestion of ctDNA with *Bgl* I. Tracks: a and j, molecular size markers (shown in kbp); b–i, 0.5 μg of ctDNA digested with 1 unit of *Bgl* I for 0.5, 2, 4, 6, 8, 12, 20, and 80 min, respectively. Arrows show bands corresponding to the *Bgl* I-generated repeated sequence of 10 kbp and multiples of this size.

contains ribosomal RNA genes, we used plasmid pSA303, containing a portion of the inverted repeat of mustard ctDNA including ribosomal RNA genes (provided by G. Link), as a probe on blots of *Acetabularia* ctDNA digested with *Eco*RI and *Bgl* I (Fig. 7). No hybridization to the repeated sequence was detected, but a number of other signals that presumably are sequences containing ribosomal RNA genes were apparent. To examine if the repeated sequence has any sequence homology with spinach ctDNA or ctDNA from the related species *A. crenulata*, we performed hybridizations using nick-translated pAmc3 at low stringency. No signals were detected with either spinach or *A. crenulata* DNA after 10 days of exposure (data not shown). This suggests that the repeated sequence is species specific. Hybridization of pAmc3 to an *E. coli* blot showed one faint signal. This, however, may be the chance homology of a small DNA sequence, since large amounts of probe and long exposure times were used.

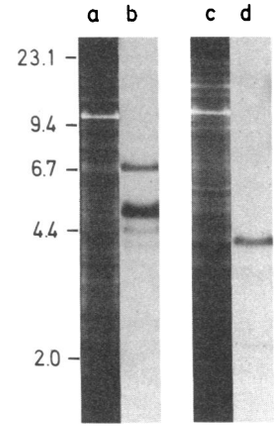

FIG. 7. Agarose gel electrophoresis of ctDNA restriction fragments and hybridization of the ribosomal probe to filter-bound DNA. *A. mediterranea* ctDNA was digested with *Bgl* I and *Eco*RI and electrophoresed on a 0.7% agarose gel. The DNA fragments were transferred to a nitrocellulose filter for hybridization. Tracks: a, photograph of *Bgl* I digest; c, photograph of *Eco*RI digest; b and d, corresponding autoradiographs of the filter hybridized to the ^{32}P-labeled fragment containing ribosomal genes. The position of molecular size markers (shown in kbp) is indicated by the numbers next to track a.

DISCUSSION

The chloroplast genome of *Acetabularia* is apparently more complex than the chloroplast genomes of other plants so far described. From the restriction analysis of the ctDNA, the size of the genome appears significantly larger than 400 kbp (unpublished data), which is more than twice the size of the largest chloroplast genome described. Circular DNA molecules have not been demonstrated for *Acetabularia*, but very long lengths of DNA, well in excess of 200 μm, have been described (7). The chloroplast genome of *Acetabularia* may consist of a very large circular molecule, but until such molecules are visualized in the electron microscope or a circular restriction map is constructed, it will remain a matter of speculation.

A large repeating sequence in the plastome of *Acetabularia* is described in this paper. It appears to be species specific because no hybridization with spinach or *A. crenulata* ctDNA was detected. The sequence appears to be present at least once as a tandem repeat of 5 or more units. The only large repeated sequence in chloroplast genomes examined contains ribosomal genes. Some low-level cross-hybridization between restriction fragments has been observed in *Chlamydomonas* (3), tobacco (23), and corn (24), but these appear to be due to small sequence homologies, and in the case of *Chlamydomonas*, it is due to the presence of 0.1- to 0.3-kbp repeated sequences (25).

We have not determined if all of the 10-kbp repeated sequences are identical, but all of the restriction enzymes that were used and that cut the sequence produce digestion products that add up to 10 kbp. This naturally does not exclude the possibility of some differences between the repeating elements. Unless some form of copy correction is operating, similar to what is known for the inverted repeats in other plastomes (5), it might be expected that some difference occurs between repeated sequences. We have no information about the physical organization of the chloroplast genome of *Acetabularia* as yet. We do know from preliminary hybridization experiments, however, that it contains ribosomal genes and other chloroplast genes such as a ribulose bisphosphate carboxylase gene (unpublished data). It might be expected that *Acetabularia* will be found to contain all of the genes described for other plastomes. If this conservative part of the plastome amounts to 100 kbp, this will leave a large part of the genome with no accountable function. In the present study we can account for at least 50 kbp as a 10-kbp repeated sequence. This would suggest that more than half of the plastome may contain new DNA sequences that need to be analyzed.

Chloroplasts from cells at a stage prior to cap formation incorporate amino acids into much the same pattern of proteins as higher plants (26, 27). This seems to argue against a coding function for the additional DNA in *Acetabularia* compared with higher plants. It is conceivable, however, that some of this extra DNA might be expressed at a different stage of the life cycle of *Acetabularia*. There is no direct experimental data to support this, but a number of enzymes that appear to be translated on 70S ribosomes, including a thymidine kinase (28), a dCMP deaminase (29), a thymidylate kinase (30), and a ribonucleoside reductase (unpublished data), are switched on during the early generative phase in *Acetabularia*.

One hypothesis worth testing is that the plastome of Dasycladaceae may contain genes that are located in the nucleus of more "advanced" plants and that have been translocated from the chloroplast to the nuclear genome. The plastome of Dasycladaceae may resemble the large ancient precursor of the modern plastome before the transfer of genes to the nucleus.

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