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Rolling-Circle Replication of Mitochondrial DNA in the Higher Plant *Chenopodium album* (L.)

STEFFEN BACKERT,[1]† PETER DÖRFEL,[1]‡ RUDI LURZ,[2] AND THOMAS BÖRNER[1]*

Institut für Biologie, Humboldt-Universität zu Berlin, 10115 Berlin,[1] and Abteilung Trautner, Max-Planck-Institut für Molekulare Genetik, 14195 Berlin,[2] Germany

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The mitochondrial genomes of higher plants are larger and more complex than those of all other groups of organisms. We have studied the in vivo replication of chromosomal and plasmid mitochondrial DNAs prepared from a suspension culture and whole plants of the dicotyledonous higher plant *Chenopodium album* (L.). Electron microscopic studies revealed sigma-shaped, linear, and open circular molecules (subgenomic circles) of variable size as well as a minicircular plasmid of 1.3 kb (mp1). The distribution of single-stranded mitochondrial DNA in the sigma structures and the detection of entirely single-stranded molecules indicate a rolling-circle type of replication of plasmid mp1 and subgenomic circles. About half of the sigma-like molecules had tails exceeding the lengths of the corresponding circle, suggesting the formation of concatemers. Two replication origins (nicking sites) could be identified on mp1 by electron microscopy and by a new approach based on the mapping of restriction fragments representing the identical 5′ ends of the tails of sigma-like molecules. These data provide, for the first time, evidence for a rolling-circle mode of replication in the mitochondria of higher plants.

The structures and modes of mitochondrial DNA (mtDNA) replication differ greatly between and within the kingdoms of eukaryotic organisms (16). Mechanisms of mtDNA replication have been described in detail for vertebrates and the baker's yeast *Saccharomyces cerevisiae* (27, 32). The mammalian mitochondrial genome consists of a unique population of supercoiled duplex molecules of 16 kb and replicates by a unidirectional theta mechanism. However, the mtDNA of trypanosomatids is organized differently; it is present as giant catenanes of minicircles and maxicircles, with the individual molecules also replicating unidirectionally via theta-like intermediates (9). The replication of the linear mitochondrial genomes of *Paramecium aurelia* and certain fungi proceeds via lariat-shaped molecules (13, 17). The mtDNA of *S. cerevisiae* is about five times larger than mammalian mtDNA. *S. cerevisiae* mtDNA contains at least five active replication origins where bidirectional replication is initiated. It has been proposed that mtDNA of *S. cerevisiae* can replicate by both theta and sigma (rolling-circle) mechanisms (32). A rolling-circle mode of replication has also been reported for the yeasts *Schizosaccharomyces pombe* and *Torulopsis glabrata* and has been suggested to be responsible for the unexpectedly large amounts of large linear molecules in preparations of mtDNA from several fungi, including *S. cerevisiae* (18, 27). Thus, detailed knowledge about the mode of replication may help to understand the in organello organization of the mitochondrial genome in yeasts as well as in higher plants.

Molecular analysis of plant mitochondrial genomes has been slow in comparison with that for other eukaryotes. This lag phase has been partly due to technical difficulties encountered in isolating mitochondria and their nucleic acids from plant cells. The other inherent problem is posed by the enormous size and the complexity of the plant mitochondrial genome. Its size ranges from about 200 to 2,400 kb in angiosperms (39). According to physical maps, the genome of plant mitochondria consists of a large circular molecule which comprises the whole genome. This so-called master chromosome contains one or more pairs of long repeats in most of the species analyzed. Homologous recombination between these repeats is thought to produce subgenomic circles (1, 26, 29). In addition to the chromosomal fraction of DNA, mitochondria may contain plasmid-like DNAs (8). The presence of the master chromosome in vivo has been the cause of much discussion. Molecules of the expected size could not be unequivocally identified by either pulsed-field gel electrophoresis or electron microscopy (EM) (1, 5). Defined size classes of subgenomic circles were not observed or were present only in minor quantities, whereas subgenomic circles of highly variable size have often been reported to occur in plant mitochondria. Typically, a high percentage of linear molecules was revealed by pulsed-field gel electrophoresis and EM in preparations of mtDNA from higher plants. Recent data strongly suggest that a major part of the mtDNA forms large, complex structures (4, 6, 28).

To date, almost nothing is known about the replication of the complex genome in plant mitochondria. Cytological studies on roots of *Pelargonium zonale* indicated that mtDNA replication may occur only in cells of a specific region near the quiescent center of the root meristem (24). Wahleithner and Wolstenholme (37) characterized the replication origins of circular mitochondrial plasmids from *Vicia faba* by DNA sequencing and by EM studies. The results were consistent with unidirectional theta replication. Two short stretches of chromosomal mtDNA harboring putative replication origins have been identified by an in vitro assay (10). These sequences showed a certain degree of homology with the H- and L-strand origins of mammalian mtDNA, but it remains to be shown if they function as origins of replication in organello. When mtDNA of cultured tobacco cells was pulse-labelled with [^{3}H]thymidine and separated by pulsed-field gel electrophoresis, incorpora-

* Corresponding author. Mailing address: Institut für Biologie (Genetik), Humboldt-Universität zu Berlin, Chausseestrasse 117, D-10115 Berlin, Germany. Phone: 49-30-20938140. Fax: 49-30-20938141. Electronic mail address: Thomas=Boerner@rz.hu-berlin.de.

† Present address: Dept. of Botany and Microbiology, Auburn University, Auburn, AL 36849-4507.

‡ Present address: Botanisches Institut, Ludwig-Maximilians-Universität München, 80638 Munich, Germany.

tion was observed primarily in the well-bound fraction and appeared only after a chase period in the fraction of mobile linear mtDNA molecules (28). There are no data available on the mode of replication of chromosomal mtDNA of higher plants or on the number and distribution of replication origins. Theoretically, the possibility exists that only the putative master chromosome is replicated and subgenomic molecules are generated exclusively by recombinational processes. Alternatively, subgenomic molecules (linear or circular) may possess replication origins and be autonomously replicating entities.

During recent EM studies on the structural organization of mtDNA isolated from a suspension culture or whole plants of *Chenopodium album*, we observed heterogenous populations of linear molecules (56.5 to 81.5% of the total number of molecules), linear molecules with branches (0.2 to 1.5%), rosette-like structures (4 to 16%), and open circles (subgenomic molecules, 13 to 26%), including circles with linear tails (4). The latter sigma-like molecules might represent intermediates of rolling-circle replication. Other putative replication intermediates (e.g., bubbles or D loops) could not be detected. A large part of the sigma-like molecules consisted of molecules corresponding in size to the 1.3-kb plasmid mp1 (14). This plasmid occurs in a high copy number in the mitochondria of cultured cells of *C. album*. Hybridization experiments revealed no homology to the chromosomal mtDNA. We determined the sequence of mp1 (1,309 bp; accession no. X58911 in the EMBL data library).

Although sigma-like DNA molecules are usually regarded as intermediates of rolling-circle replication (15), they may also be generated by breakage of intermediates of a theta type of replication (9) or, theoretically, by recombinational processes between circular and linear molecules (36). Therefore, the aim of the present study was to further elucidate the mode of replication of the mtDNA of a higher plant. We observed stretches of single-stranded DNA (ssDNA) in sigma-like molecules and even entirely single-stranded molecules of the plasmid and of subgenomic molecules by Southern analyses and by EM studies with single-strand-binding (SSB) proteins. Moreover, we have mapped origins of replication on the circular plasmid mp1. Our results indicated that mp1, and probably the subgenomic circular DNA molecules in the mitochondria of *C. album*, are replicated by a rolling-circle mode via sigma-like intermediates.

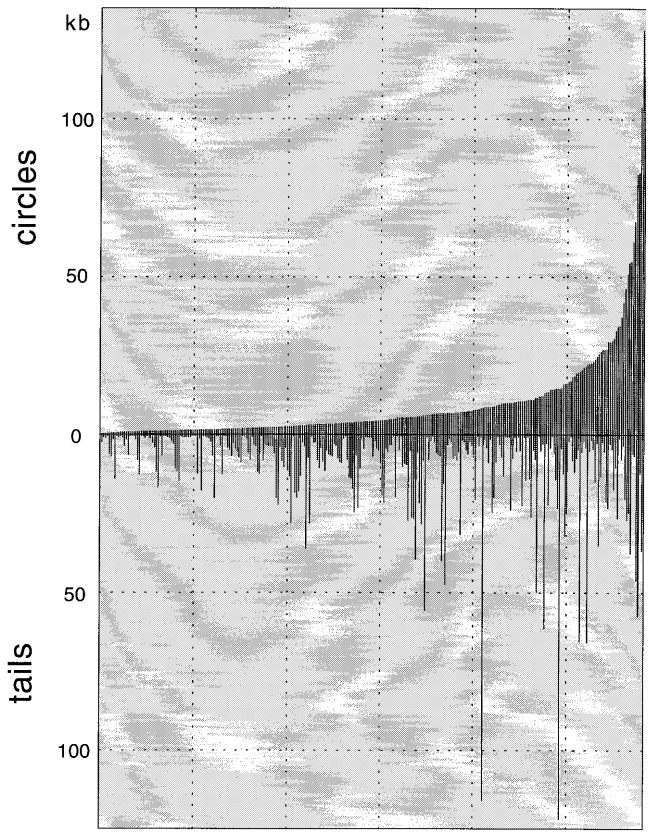

FIG. 1. Size distribution of 291 sigma-like structures from 8,000 mtDNA molecules of *C. album* suspension cultures (logarithmic growth stage) analyzed by EM. Each bar represents one molecule consisting of a circle with a corresponding tail. The first four molecules (from the left) were smaller than the plasmid mp1, which itself is not included in the figure.

MATERIALS AND METHODS

Plant material. Mitochondria were isolated from whole plants of *C. album* (3 weeks old; collected at the Botanical Garden, University of Potsdam, Germany) and from the related suspension culture C.9.1. The conditions of cultivation have been described earlier (14). Cells were harvested in the early phase of growth (1 and 3 days after transfer into new medium), in the logarithmic growth stage (6 days), and in the early (9 days) and late (12 days) stationary growth phases (21).

Preparation of mtDNA. Mitochondria were purified by differential centrifugation, treated with DNase I, centrifuged on a discontinuous gradient, and collected from the 52%–30% interphase (33). The mitochondria were lysed by addition of 0.1 mg of proteinase K per ml and 1% sarcosyl followed by incubation at 50°C for 1 h. mtDNA was isolated by phenol-chloroform extraction and ethanol precipitation (30).

Preparation of bacterial DNA and plasmid sequencing. The mitochondrial plasmid mp1 was cut with *Bam*HI, ligated into vector pGEM3zf(+) (Promega, Madison, Wis.), and transformed into *Escherichia coli* XL-1 Blue (30). Total *E. coli* DNA (including the recombinant plasmid mp1) was prepared by precipitation of cells (harvested after growth for 4 h in Luria-Bertani medium by centrifugation at 2,000 × *g*) followed by DNA extraction as described above. Plasmid mp1 was purified by using the plasmid extraction kit from Qiagen (Düsseldorf, Germany) and sequenced by chain termination (31) with the Sequenase version 2.0 DNA sequencing kit from U.S. Biochemicals (Bad Homburg, Germany). Computer analysis of the mp1 sequence was done with the PC Gene program (release 6.5). Homology searches were performed with the EMBL, Gene Bank, Swiss Prot, and PIR data banks.

Enzymatic digestions, agarose gel electrophoresis, blotting, and hybridization. Digestion of mtDNA by restriction enzymes and S1 nuclease was performed as recommended by the supplier (Amersham-Buchler, Braunschweig, Germany). Conventional agarose gel electrophoresis of DNA was done for 8 to 10 h at 5 V/cm in 1.5% agarose gels in 1× Tris-borate-EDTA electrophoresis buffer with or without ethidium bromide (0.3 μg/ml) (see figure legends) in a large electrophoresis chamber (model HRH; IBI, New Haven, Conn.). After electrophoresis, the DNA was stained with ethidium bromide (if not already present during the run), photographed, and blotted by alkaline transfer to Zeta Probe GT membranes according to the instructions of the supplier (Bio-Rad, Richmond, Calif.). The following mitochondrial probes were used for hybridization: plasmid mp1 (cut out of the vector) and a construct consisting of vector and mp1. Radioactive labelling of the probes was performed with the Rediprime kit and 1.85 MBq of [α-^{32}P]dCTP, provided by Amersham-Buchler. Filters were hybridized overnight in 6 to 8 ml of 7% sodium dodecyl sulfate–250 mM NaH_2PO_4 (pH 7.2) at 65°C in hybridization tubes from Schott (Mainz, Germany) and then washed under stringent conditions according to standard protocols (30).

EM. For EM studies, three different approaches were employed. First, for characterization of the plasmid mp1, mtDNA was prepared as described above and then separated on a 0.4% agarose gel. Pieces of agarose containing DNA molecules ranging in size from about 0.5 to 6 kb were excised from the gel. DNA was by electroeluted into a dialysis bag and purified by extraction with phenol-chloroform (1:1) and chloroform followed by ethanol precipitation (30). Plasmid molecules were prepared for EM by resuspension in carbonate buffer as described previously (34).

Second, in order to minimize the danger of mechanical breakage of large molecules in preparations of total mtDNA, we applied the following gentle procedure. Purified mitochondria were immobilized in low-melting-point (LMP) agarose, lysed, and then washed according to a standard protocol (19). Contaminating RNA was removed by digestion with the RNase cocktail of Stratagene (La Jolla, Calif.) (3). The agarose was then digested with gelase according to the protocols of the supplier (Biozym, Hameln, Germany). Remaining undigested LMP agarose was pelleted by centrifugation for 5 min at 2,000 × *g*. The supernatant containing total mtDNA was prepared for EM analyses by the droplet method (25).

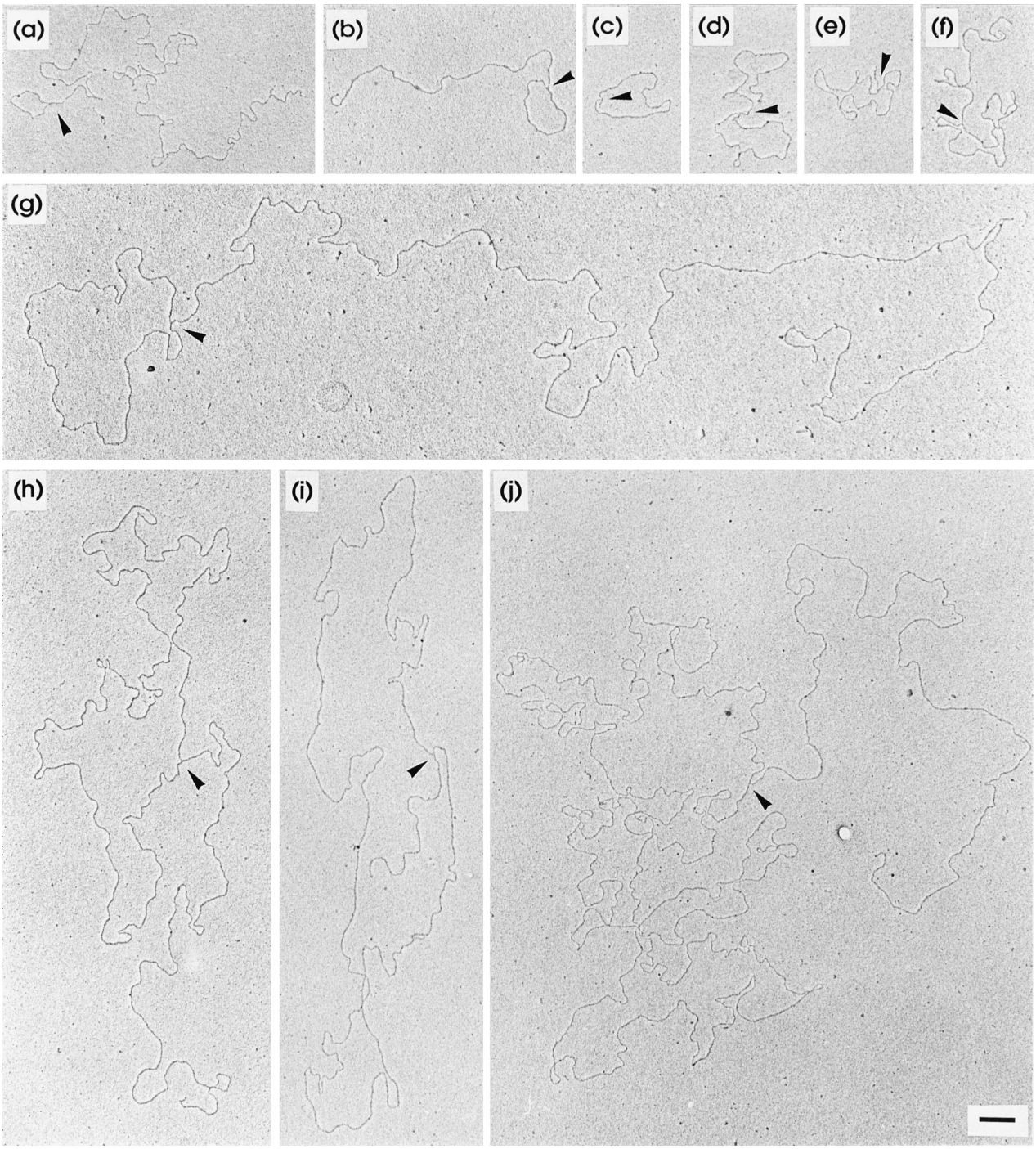

FIG. 2. Electron micrographs of 10 sigma-like structures of different-sized subgenomic mtDNA circles of *C. album* prepared by the droplet method. (a) Circle of 3 kb, tail of 28 kb; (b) circle of 4.4 kb, tail of 9 kb; (c) circle of 6.5 kb, tail of 0.2 kb; (d) circle of 7.4 kb, tail of 6.2 kb; (e) circle of 8.9 kb, tail of 0.5 kb; (f) circle of 11.6 kb, tail of 7.9 kb; (g) circle of 20 kb, tail of 65.5 kb; (h) circle of 51 kb, tail of 26.9 kb; (i) circle of 61.1 kb, tail of 6.1 kb; (j) circle of 128 kb, tail of 36.5 kb. Arrowheads indicate the junction of the circle and tail. Bar, 1 kb.

Last, for visualization of single-stranded mtDNA stretches by coating with SSB protein from *E. coli*, we applied the mica adsorption method. About 50 ng of mtDNA isolated from the suspension culture (logarithmic growth stage) and ssDNA of phage ϕX174 as the control were incubated with SSB protein from *E. coli* (kindly provided by E. Scherzinger, Max-Planck-Institut für Molekulare Genetik, Berlin, Germany) at DNA/protein mass ratios of 1:1 to 1:10 (wt/wt) in 1× binding buffer (25 mM Tris-HCl [pH 7.5], 100 mM KCl, 10 mM $MgCl_2$) in a total volume of 50 μl for 30 min at 30°C. Nucleic acid-protein complexes were then fixed by addition of 2.5 μl of 4% glutaraldehyde. To minimize the action of shearing forces on mtDNA molecules, we did not remove unbound SSB by a chromatography step. The samples incubated with SSB as well as DNAs without added SSB (controls) were prepared for EM by the mica adsorption method (40).

A transmission electron microscope (model E400T; Philips, Eindhoven, The Netherlands) operating at 80 kV was used in all investigations. The size of mtDNA molecules was determined by comparison with the lengths of internal standards, namely, the double-stranded plasmid pBR322 (4,363 bp) and ssDNA and double-stranded DNA (dsDNA) from the phage ϕX174 (5,386 bases).

TABLE 1. Percentages of sigma-shaped molecules in circular and total mtDNAs from preparations of whole plants and suspension-cultured cells of *C. album*[a]

mtDNA	% of sigma-shaped molecules in:					
	Harvest of suspension-cultured cells after:					Plants after 3 wk
	1 day	3 days	6 days	9 days	12 days	
Circular	2.1	5.2	18	0.9	0.0	1.2
Total	0.5	1.3	4.8	0.3	0.0	0.5

[a] Four hundred individual molecules were analyzed in each fraction.

RESULTS

EM studies of chromosomal mtDNA. Preparations of mtDNA from the cell culture of *C. album* were observed to contain many more sigma-shaped molecules than preparations from whole plants, suggesting that this type of molecule occurs mainly in dividing cells (4). During this study, we measured the sizes of the circular and linear regions of 291 sigma-like molecules (found among a total of 8,000 mtDNA molecules of the suspension culture). Circles and tails showed a continuous size distribution (Fig. 1 and 2). The largest circle had a size corresponding to 128 kb with a tail of about 36.5 kb (Fig. 2j). We extended these studies and performed an EM examination of mtDNA molecules prepared from cells of the suspension culture harvested at different growth stages as well as from whole plants of *C. album*. From each preparation, 400 individual molecules were investigated. The largest amount of sigma-like molecules (4.8% of all molecules) was detected when cells were harvested 6 days after transfer of the cells into new medium, i.e., during the logarithmic phase of growth (Table 1). Therefore, all further studies were performed with mtDNA isolated from cells harvested in this phase. Moreover, we examined the junctions of the circle and tail at a higher magnification. The resulting photomicrographs show no evidence of artificially associated linear and circular molecules (Fig. 3).

Detection of single-stranded regions in sigma-like molecules. The generation of sigma-like molecules is usually taken as an indication of the occurrence of rolling-circle replication (11, 12, 15, 27), although intermediates of the replication of linear mitochondrial genomes have a similar appearance in EM (13, 17). Rolling-circle replication, via sigma-like intermediates, is the only process for which the generation of circular ssDNA molecules has been convincingly demonstrated (2, 11, 12, 35). Therefore, we searched for single-stranded regions in the sigma-like structures and entirely single-stranded molecules within the *C. album* mitochondria.

mtDNA was initially prepared from the suspension culture, separated in 0.4% agarose gels, and eluted from gel regions containing plasmid mp1 and its oligomeric forms. The eluted DNA was prepared for EM investigation by spreading with carbonate buffer in the presence of formamide, a method usually used to distinguish between ssDNA and dsDNA forms by the thicknesses of their contours (34). Analysis of 1,000 molecules, which according to their size should represent the plasmid mp1, revealed that the majority of plasmid molecules consisted of open circles (among them a few were catenane-like [Fig. 4i]) and supercoiled molecules. We detected 53 sigma-like molecules with a circle of about 1.3 kb (monomer size) with either single-stranded (9 molecules) or double-stranded (44 molecules) tails. The length of the single-stranded tails varied from about 120 to 1,000 bases (examples are shown in Fig. 4a and b), whereas the double-stranded tails ranged from about 100 bp to about 5 kb (Fig. 4c to g; Fig. 5). Nine putative di- or trimers of mp1 with single- or double-stranded tails were identified on the basis of their sizes (not shown). Additionally, we observed entirely single-stranded molecules. These comprised 13 circular and two linear mp1 monomers (Fig. 4h) and two single-stranded circular molecules of 2.6 kb which could represent single-stranded dimers of mp1.

In order to visualize stretches of ssDNA in sigma-like molecules, we used, as another approach, SSB proteins (Fig. 6). SSB-mtDNA complexes with a smooth contoured appearance and relatively low background level were observed at a DNA/protein ratio of 1:5 (wt/wt). The ssDNA-SSB complexes had a contour length of 0.345 μm (±0.03 μm) in the case of φX174 ssDNA (100 measurements), corresponding to about 20% of the length of SSB-free φX174 dsDNA standards (1.725 μm) (not shown). A total of 1,400 individual mtDNA molecules were analyzed, and the distribution of sizes and types (linear, circular, and complex) of molecules was similar to that previously observed (4). Among the examined molecules, we found 25 sigma-like molecules. Twenty-one of these had single-stranded regions at the ends of their tails (Fig. 6a to d, small arrowheads). Moreover, one or two beads of SSB protein, indicating short ssDNA stretches, were found at the junction of the ring and the tail (Fig. 6b, d, and e, large arrowheads), and in a few cases, longer single-stranded regions were detected within the circle close to the junction (Fig. 6c, large arrowhead). Four sigma-like molecules had no SSB protein either at the circle-tail junction or at the end of the tail (Fig. 6a and e). The ssDNA stretches of these molecules might have been too short to allow binding by the SSB protein. The SSB protein of *E. coli* binds as a tetramer which requires at least 64 nucleotides for attachment, and so shorter stretches cannot be detected by the SSB-binding assay (23). Additionally, 20 single-stranded circular mtDNA molecules which were uniformly covered by SSB protein were observed. Of these molecules, six were present as monomers and three were present as putative dimers of the plasmid mp1. The other circles had sizes of 2.0, 2.9, 4.6, 5.5, 7.1, 7.5, 8.4, 9.4, 11.2, 15.6, and 18.7 kb. Examples are shown in Fig. 6f to i.

Detection of single-stranded plasmid DNA by hybridization. To obtain further evidence for the existence of entirely single-stranded mp1 molecules, we separated mtDNA electrophoretically in agarose gels and followed this with alkaline transfer to a nylon membrane (Fig. 7). If undigested, mp1 was found after hybridization at positions in the gel corresponding to monomers in linear, open circular, and supercoiled forms. Oligomers of mp1 were detected, as well as signals in the slot (Fig. 7b, lane 2). Additionally, a very faint spot could be observed in front of the supercoiled form after long exposure times (Fig. 7c, lane 2). Signals at this position result from entirely single-stranded molecules (35) as demonstrated here for single- and double-stranded M13 DNAs (Fig. 7a, lanes 6 and 7). The signal observed at this position after hybridization with mp1 should therefore also represent the single-stranded form of this plasmid. This conclusion is supported by the observation that this signal disappeared after digestion with S1 nuclease (Fig. 7a, lane 3) but not when mtDNA was digested with restriction endonucleases (lanes 4 and 5). Digestion with *Bam*HI linearizes mp1 (Fig. 7a, lane 4), and *Eco*RI cuts the plasmid into two pieces of about 1.1 and 0.2 kb (lane 5). Interestingly, S1 nuclease treatment also resulted in the disappearance of well-bound plasmid DNA and transformed a small part of the supercoiled form of mp1 into open circles and linear molecules. As a control, DNA isolated from *E. coli* under the same conditions contained only supercoiled and open circular monomeric forms of the plasmid mp1 (integrated

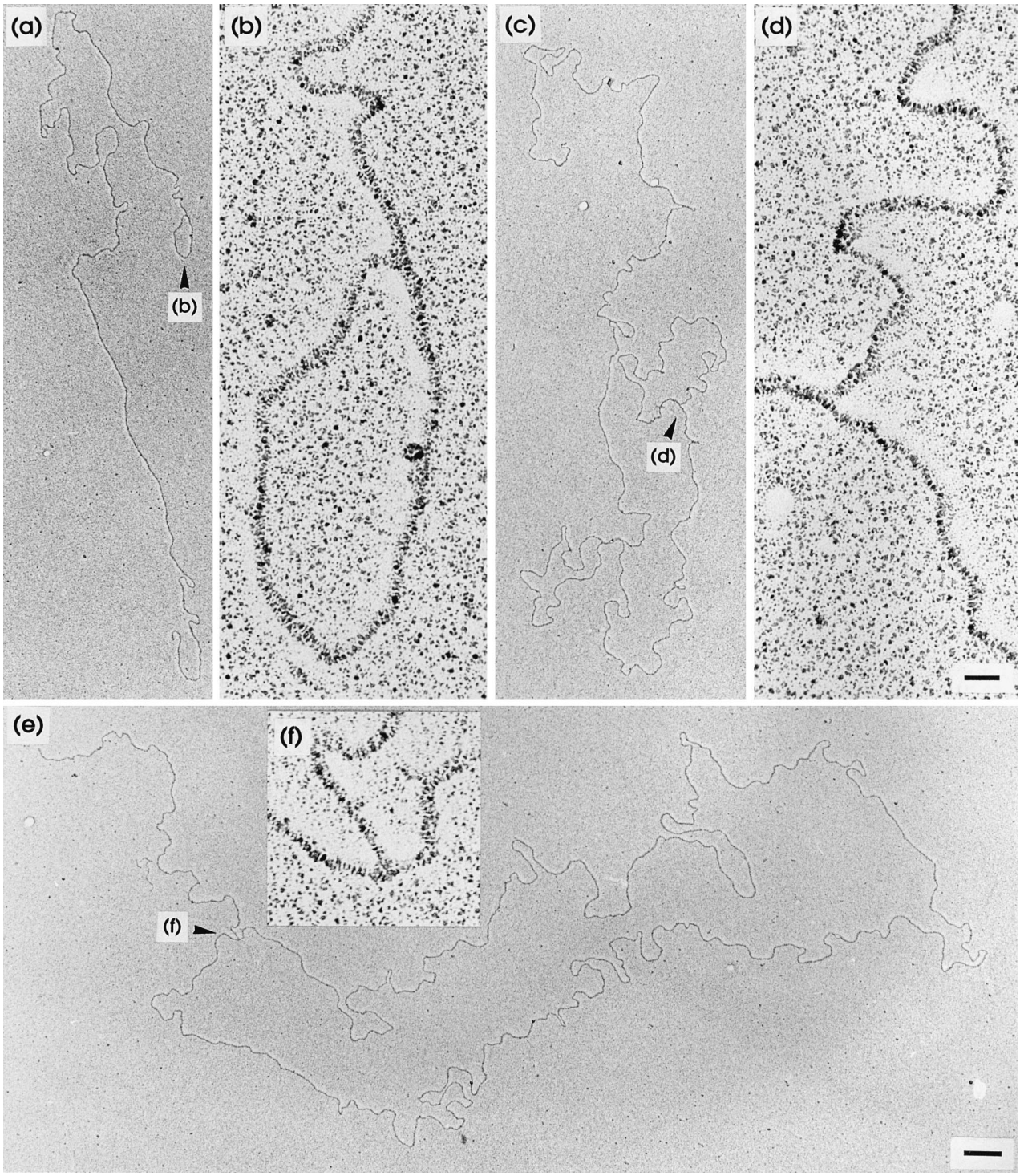

FIG. 3. EM analysis of the circle-tail junctions (b, d, and f) by using approximately 10-fold magnification of three different-sized rolling circles (a, c, and e) of chromosomal mtDNA from *C. album* prepared by the droplet method. (a) Circle of 2.9 kb, tail of 36 kb; (c) circle of 13.1 kb, tail of 61.4 kb; (e) circle of 104 kb, tail of 12.5 kb. The bar in panel e represents 1 kb for panels a, c, and e; the bar in panel d represents 0.1 kb for the higher magnifications in panels b, d, and f.

in a vector), with no oligomers or well-bound or single-stranded forms present (Fig. 7a and d, lanes 9).

Identification of possible replication origins. Cutting of replication intermediates by restriction endonucleases generates entirely different molecules depending on whether intermediates are of the theta type (i.e., circles with replication bubbles) or the sigma type (i.e., rolling circles) (Fig. 8a). To obtain evidence for a sigma type of replication and to localize the origin(s) of replication, mtDNA was digested with *Mlu*I, *Acc*I, and *Pst*I, electrophoretically separated in a conventional neutral agarose gel, blotted, and hybridized with the plasmid mp1 as a probe (Fig. 8b to d). As expected, the strongest signal

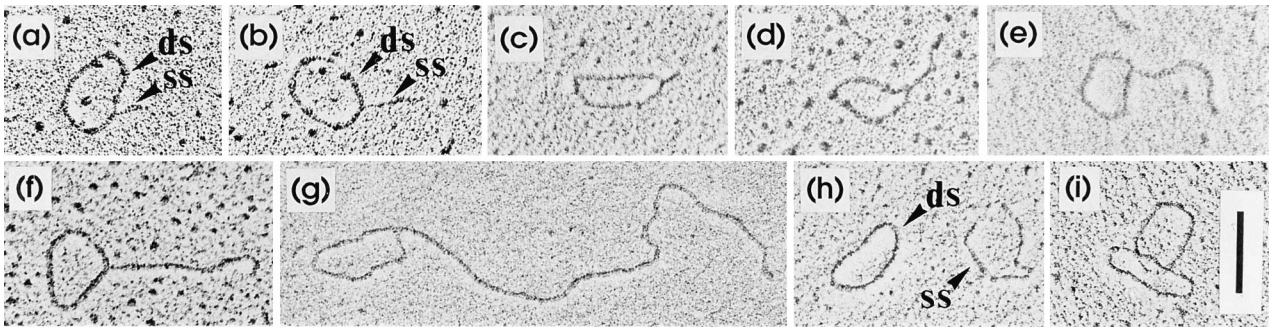

FIG. 4. Electron micrographs of different forms of plasmid mp1 from *C. album* obtained by spreading in carbonate buffer. (a to g) Typical sigma-like structures with the size of mp1. (a) Double-stranded circular monomer (1.3 kb) with a single-stranded tail (170 bases); (b) double-stranded circular monomer with a single-stranded tail of 400 bases; (c to g) double-stranded circles of 1.3 kb with double-stranded tails of 0.1 kb (c), 0.6 kb (d), 0.9 kb (e), 1.2 kb (f), and 3.4 kb (g). (h) One double-stranded circle and one single-stranded circle of 1.3 kb; (i) two double-stranded circles of 1.3 kb interlocked as a catenane. Bar, 1 kb.

appeared at the position of the linear monomer. However, signals were also found at lower mobility and in the well, which might correspond to larger single-stranded plasmid forms and replication intermediates with single-stranded regions. Additionally, a ladder of distinct fragments with sizes smaller than the monomer was observed (Fig. 8c), as well as a second ladder of still smaller fragments after long exposure times (Fig. 8d). Such distinct fragments could result from cutting the double-stranded tail of sigma-like molecules but not from digestion of intermediates of theta-type replication (Fig. 8a) and therefore should be useful to localize so-called double-stranded origins (*dso*) of rolling-circle replication. These fragments could indicate the site of action of an endonuclease which might nick one strand of the circle (hereafter called the leading strand), thereby initiating replication via rolling circles. We projected the two pairs of small fragments on the map of mp1 starting from the cutting site of the respective restriction endonuclease. All fragments of the first ladder ended at around nucleotide position 730, and the fragments of the second ladder ended at approximately position 540 (Fig. 8e). These positions were designated plasmid replication origins *dso 1* and *dso 2*. From these data one can also predict the direction of leading-strand replication, which was identical for both origins (Fig. 8e). The accuracy of this *dso* mapping was proven by hybridization of the same blots by using a set of distinct restriction fragments of mp1 as probes (Fig. 8e). The *Mlu*I-*Acc*I fragment hybridized only with the small fragments in lanes 3 of Fig. 8b and c, while the *Mlu*I-*Pst*I fragment hybridized only with the small fragments in lanes 3 and 4. A *Bam*HI-*Bgl*I fragment which is situated outside the region covered by the two ladders of three short fragments gave no hybridization signals (not shown). The nucleotide sequences around the two origins have six base pairs in common but no homology to known sequences of origins of rolling-circle replication. The small open reading frames found on the plasmid did not show significant homologies to known genes, including genes coding for a proposed nicking-closing enzyme involved in rolling-circle replication of bacterial plasmids or phage genomes (2, 12).

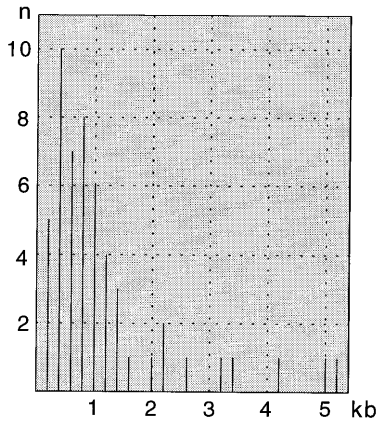

FIG. 5. Distribution of tail lengths from 53 sigma-like plasmid mp1 molecules found in the suspension culture of *C. album*. The figure includes nine mp1 circles with single-stranded tails of 0.1, 0.17, 0.2, 0.25, 0.38, 0.4, 0.73, 0.9, and 1.0 kb.

To further verify the localization of the origins, we linearized DNA of the 0.5- to 6-kb fraction (eluted from gels) by cutting with restriction endonucleases *Pvu*II, *Sma*I, and *Bgl*I and prepared it for EM. Altogether, 112 branched molecules with a Y shape, which should result from cutting sigma-like mp1 molecules, were photographed and measured. Thirty-four Y-shaped molecules were asymmetric and therefore could be used for mapping of the origins (Table 2). We deduced the location of the origins (*dso*) from the lengths of the arms of such molecules and the known positions of the restriction sites. The position of the putative *dso*, determined from two-thirds of all Y-shaped molecules checked, was in the region of nucleotides 700 to 750, which is in good agreement with the determination of the position of *dso 1* and the direction of replication by use of the small restriction fragments. The putative origin of seven other Y-shaped molecules was determined to be 180 to 200 bp upstream (against the direction of replication, position 510 to 560), which fits the position of *dso 2* determined by the small-fragment approach. The remaining four asymmetric Y-shaped molecules did not indicate a common site as an origin. It is possible that molecules were erroneously identified as mp1 as a result of plasmid DNA preparations containing low levels of small circles of chromosomal mtDNA with sizes similar to that of mp1. Additionally, six dual branchings on the monomer (double Y's) were observed. These molecules were also mapped. The lengths of their branches were in agreement with the presumptions that restarts can occur at the origins and that both origins can be used simultaneously for the initiation of rolling-circle replication.

DISCUSSION

One of the difficulties encountered in studying plant mtDNA replication is the enormous size and complexity of the genome and the lack of a specific size class or classes of circular DNA species. We tried to overcome some of these problems by

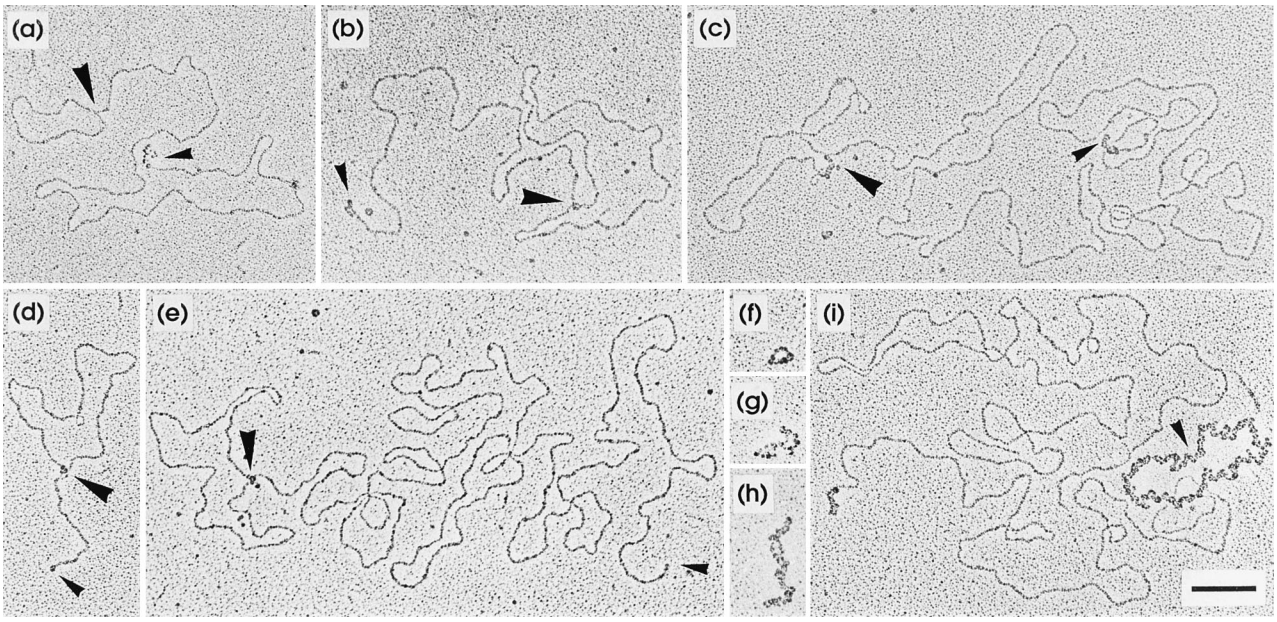

FIG. 6. Electron micrographs of replication intermediates (sigma-like molecules [a to e] and ssDNA circles [f to i]) of chromosomal mtDNA from a *C. album* suspension culture. Stretches of ssDNA are coated with SSB protein from *E. coli*. (a) Double-stranded circle of 2 kb, double-stranded tail of 10 kb including a single-stranded region of 1,800 bases at the end of the tail (small arrowhead); (b) double-stranded circle of 5.8 kb, double-stranded tail of 6.35 kb with a short single-stranded stretch (about 100 bases) at the circle-tail junction (large arrowhead) and a single-stranded stretch of 740 bases at the end of the tail (small arrowhead); (c) double-stranded circle of 5.8 kb with a single-stranded stretch of 1,200 bases on the lagging strand of the circle (large arrowhead), tail of 21.6 kb including a ssDNA region of 1,000 bases at the end (small arrowhead); (d) double-stranded circle of 4.2 kb with a double-stranded tail of 1.7 kb with stretches of ssDNA (100 bases each) at the circle-tail junction (large arrowhead) and at the end of the tail (small arrowhead); (e) double-stranded circle of 5.5 kb with a tail of 27.2 kb, the single-stranded circle-tail junction is coated with one bead of SSB protein (large arrowhead), no ssDNA is detectable at the end of the tail (small arrowhead); (f) 2 kb; (g) 4.6 kb; (h) 9.4 kb; (i) among two linear molecules is an 18.7-kb ssDNA circle (arrowhead). Bar, 0.5-kb dsDNA and 2.5-kb ssDNA.

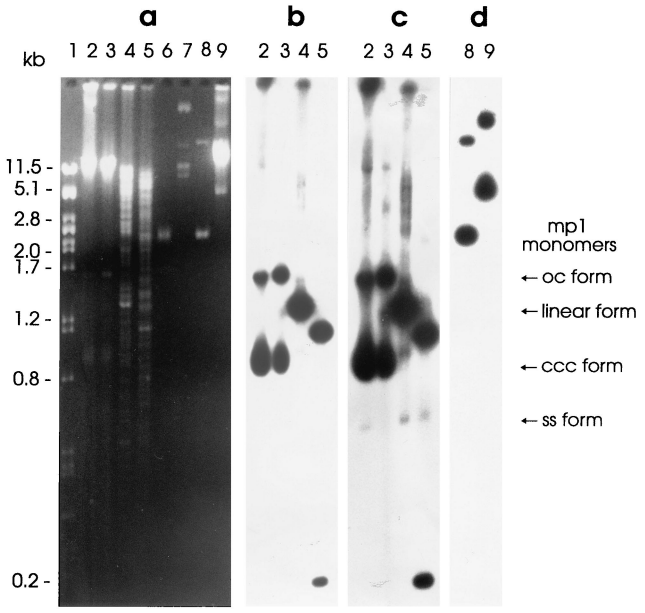

FIG. 7. Identification of entirely single-stranded plasmid mp1 forms by Southern blot analysis. Agarose gel electrophoresis was performed in the presence of ethidium bromide as described in Materials and Methods. Lanes: 1, λ DNA digested with *Pst*I; 2, mtDNA, untreated; 3, mtDNA treated with nuclease S1; 4, mtDNA digested with *Bam*HI; 5, mtDNA digested with *Eco*RI; 6, M13 ssDNA; 7, M13 dsDNA; 8, vector pGEM3zf(+); 9, total *E. coli* DNA including cloned mp1 in vector pGEM3zf(+). Arrows indicate the positions of monomeric mp1 forms in the gel (oc, open circular; ccc, covalently closed circular; ss, single stranded). The hybridization probe was mp1 (b and c) and mp1 in vector pGEM3zf(+) (d). Exposure times were 10 h for panel b and 25 h for panels c and d.

examining a circular plasmid as a model for mtDNA replication. The plasmid and the chromosomal mtDNAs replicate in the same compartment, and thus they may use the same arsenal of replication proteins. The results of the present study provide, for the first time, evidence for the occurrence of rolling-circle replication in the mitochondria of higher plants. The existence of this type of replication may have impacts on understanding the structure and function of the plant mitochondrial genome.

Results obtained by several entirely different approaches led to the conclusion that the plasmid mp1 is amplified via a rolling-circle mechanism. First, we could show that a large number of circles, of the size of mp1, exist as sigma-like molecules. The relative number of sigma-like molecules was much lower in preparations from whole plants and from a suspension culture in stationary phase than in preparations from actively dividing cells (Table 1). This difference suggests a functional importance of these molecules and makes preparation artifacts as a possible source of sigma-like molecules highly unlikely. Circular molecules with linear tails are generally regarded as typical intermediates of rolling-circle replication (12, 15, 27). Similar molecules might theoretically also originate from breakage of theta-like intermediates of replication or from recombining molecules. However, it is very unlikely that the observed sigma-like molecules from *C. album* mitochondria were generated from breakage of theta intermediates. Up to now we have analyzed approximately 20,000 individual DNA molecules from 22 independent preparations of mitochondria from cultured cells or whole plants by EM. We never observed linear or circular molecules with replication bubbles expected from theta-type replication. If the sigma-form molecules originated from such hypothetical theta-like molecules, then all molecules with a replication bubble must have broken exactly

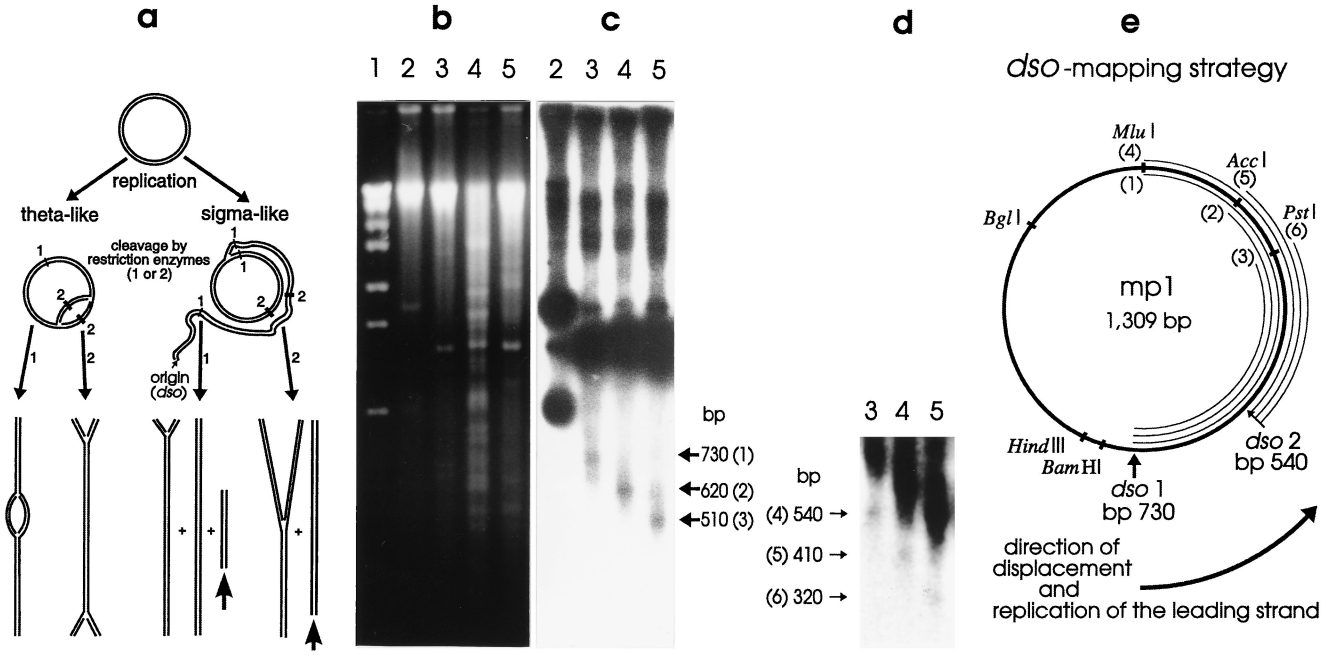

FIG. 8. New approach for mapping an origin of rolling-circle replication. (a) Molecules resulting from digestion of different types of replication intermediates of a given circular molecule by restriction endonucleases. Digestion of a hypothetical theta-like structure results in only one molecule, whereas sigma-like structures are cut into several fragments depending on the length of the tail. Provided that rolling-circle replication is initiated by nicking at a specific sequence, cutting of the double-stranded tail produces small fragments representing the sequence between the nicking site (*dso*) and the restriction site (arrows). (b) Ethidium bromide-stained neutral agarose *dso* mapping gel of plasmid mp1. (c and d) Autoradiographs after hybridization with cloned plasmid DNA after exposure times of 20 h (c) and 50 h (d). mtDNAs from a *C. album* suspension culture undigested (lanes 2) or digested with *Mlu*I (lanes 3), *Acc*I (lanes 4), and *Pst*I (lanes 5) are shown. DNA digested with *Eco*T14I (lane 1) and λ DNA digested with *Pst*I (not shown) were used as size markers. (e) The low-molecular-weight fragments (indicated by arrows in panels c and d) projected on the circular map of mp1 end around position 730 (*dso 1*) and position 540 (*dso 2*). In these regions rolling-circle replication of the leading strand starts and proceeds in the indicated direction.

at the replication fork, as depicted in Fig. 9a. This would also indicate that the circular part of the sigma-like molecules observed in our preparations represented a replication bubble. In this case, one would not expect to find a major portion of all bubbles having exactly the size of mp1. Moreover, the generation of circles with one tail which is longer than its circumference (as found in our preparations), by breakage of a theta structure, is not possible (Fig. 9). We did not observe degradation products of theta structures as shown in Fig. 9. One single-strand break in a theta structure would generate a sigma-like molecule with single-stranded regions at the end of the tail, at the tail-circle junction, and at a definite site within the circle (Fig. 9a). However, we observed single-stranded stretches only at those sites where they should be expected from sigma-like intermediates of rolling-circle replication (36), namely, at the end of the tail and at the tail-circle junction (Fig. 6). A few circles with two tails have been observed in mtDNA preparations (4) but never with the tail lengths expected from broken replication bubbles (Fig. 9b) or with tails starting at opposite sites of the corresponding circle (Fig. 9d). Symmetric

TABLE 2. Mapping of two replication origins (*dso 1* and *dso 2*) on plasmid mp1 by linearization with restriction endonucleases and EM analysis of resulting asymmetric Y forms

Deduced *dso*	No. of asymmetric Y molecules after digestion with:		
	*Pvu*II (bp 220)	*Sma*I (bp 413)	*Bgl*I (bp 1099)
dso 1	9	7	7
dso 2	2	2	3
dso 1/2	1	2	1
dso 1/1	0	1	0
dso 2/2	1	0	0
Others	1	2	1

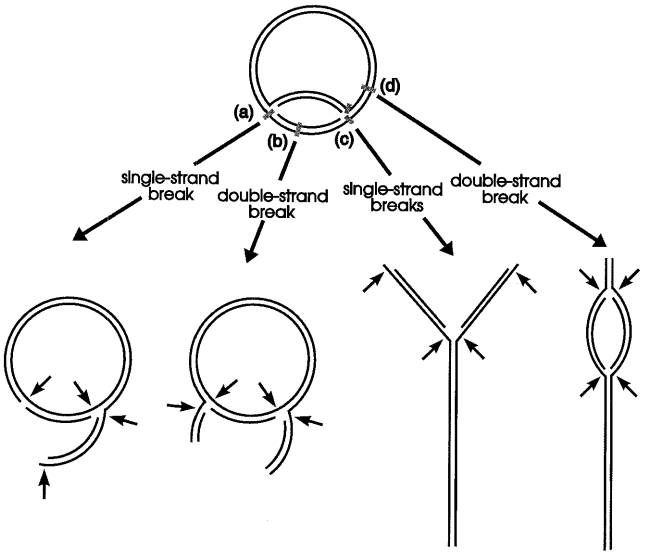

FIG. 9. Schematic representation of possible breakage products of a hypothetical theta-like structure. Arrows indicate the expected positions of single-stranded regions in these molecules.

Y-shaped structures with two identical arm lengths (Fig. 9c) were not detected among the uncut mtDNA molecules (4).

Second, we developed a new approach to demonstrate that the observed putative replication intermediates have tails with one of two identical 5′ ends which are expected exclusively from the sigma-like intermediates of rolling-circle replication and not from other types of replication or recombination. The indicative small fragments specifically hybridized with mp1 and were observed only after cutting of mp1 by restriction endonucleases, and they thus represent linear subfragments of this plasmid. The small fragments did not hybridize with a *Bam*HI-*Bgl*I fragment of mp1 as a negative control but hybridized specifically with those restriction fragments of mp1 which should contain their sequences. On the other hand, no other small fragment hybridizing to this *Bam*HI-*Bgl*I fragment could be detected, showing that these fragments should have their origin from tails displaced by rolling-circle replication and not from broken mp1 circles. Therefore, by this small-fragment approach we were able to localize one origin (*dso 1*) near position 730, according to the sequence of mp1, starting with position 1 at the unique cleavage site of *Mlu*I. A second set of small fragments hybridizing with mp1 indicated the existence of a second origin (190 bp upstream from *dso 1*), designated *dso 2*. The very weak hybridization signals obtained from these fragments suggested that *dso 2* is used as an origin less often than *dso 1*. These results could be verified by EM mapping. Two origins were tentatively localized in the same region of the plasmid. All mapping data predict that replication of mp1 is initiated by site- and strand-specific cleavage events followed by unidirectional replication starting from both origins.

Third, we detected ssDNA copies of mp1, single-stranded tails associated with sigma-shaped molecules, and single-stranded regions in monomeric and oligomeric forms of mp1. Complete single-stranded molecules are characteristic products of asymmetric rolling-circle replication. These structures result when the displaced parental strand (the tail [leading strand]) is not used as a template for replication, as known from coliphages (2) and rolling-circle-replicating plasmids (12, 35, 36), and they provide strong evidence for the occurrence of rolling-circle replication. Obviously, some mp1 molecules were replicated asymmetrically. However, as inferred from the large number of sigma-like structures consisting mostly of dsDNA, we conclude that synthesis of leading and lagging strands of mp1 is a coupled, i.e., symmetric, process in most cases.

Furthermore, we detected tails longer than the corresponding circle in about half of the sigma-like molecules. The production of concatemers is a common feature of recombination-dependent rolling-circle replication when the tails are not cut into unit length after each round of replication (36). Thus, rolling-circle replication provides, in addition to recombination, an explanation for the origin of the observed oligomeric forms of mp1. Such oligomers are known to occur with almost all small circular plasmids of plant mitochondria (8). The generation of concatemers would indicate a relaxed type of replication which is regarded to be typical for cytoplasmic genomes (7).

The model of the structural organization of the plant mitochondrial genome based on data from physical mapping predicts the existence of a master chromosome which generates a set of subgenomic circles by recombination between large repeats (26, 29). Although neither the master chromosome nor subgenomic molecules of the predicted sizes have been observed in higher plant mitochondria, the existence of smaller subgenomic circles cannot be disputed. Recent extensive studies on the structural organization of mtDNA from *C. album* did not reveal molecules of the total genome size (270 kb), alternatively describing a continuous size distribution of subgenomic circles from about 0.3 to 183 kb (4). The question arises as to whether these subgenomic circles were generated exclusively by replication and/or recombinational events. Our results suggest that subgenomic molecules of higher plant mtDNA may indeed be replicons. A high percentage (about 18%, excluding mp1) of the observed circles isolated at logarithmic growth phase from cultured cells of *C. album* had a tail, i.e., exhibited the typical sigma shape of intermediates of rolling-circle replication. The distribution of ssDNA stretches in these molecules was in agreement with their function as replication intermediates. Small amounts of the same molecules were also found in preparations from the suspension culture in early and late stages of growth and in whole plants but not in the stationary growth phase of cultured cells. Therefore, these sigma-like molecules of subgenomic size should, like the sigma-shaped mp1 molecules, indeed represent intermediates of a rolling-circle type of replication. This conclusion was strongly supported by the detection of completely single-stranded circular molecules with sizes ranging from about 1 kb up to 18.7 kb. Such ssDNA molecules were observed only as products of asymmetric rolling-circle replication (2, 11, 12, 35). Even a circle as large as 128 kb was found to have a tail (Fig. 2j) and may therefore represent a rolling circle. As in the case of sigma-like molecules with the size of mp1, almost one-half of these large sigma-shaped molecules (48.3%) had tails larger than the respective circle. It is possible, therefore, that these long tails are head-to-tail concatemers, i.e., contain more than one unit length of the parental circle. Therefore, subgenomic molecules may also exist as oligomers and contribute to the extreme variability in the size of mtDNA circles.

In light of the postulated high recombinational activity of plant mtDNA and its relatively high content of ssDNA, it is tempting to speculate that its replication might depend on recombinational activity. Initiation of replication at specific sites, as observed for mp1, however, was not found in the case of recombination-dependent rolling-circle replication in bacteria (36). The observation of a rolling-circle mode of replication in the mitochondria of *C. album* does not rule out the possibility that plant mtDNA is replicated via another mode in other species, under different conditions, or in certain cell types. It is known from bacteria that the presence or absence of the activity of a single nuclease determines if DNA molecules are replicated by the sigma or the theta type of replication (36). Some DNA molecules, like the mitochondrial genome of *S. cerevisiae* (32) and the chloroplast chromosomes (22), are reported to replicate via different types of intermediates. However, sigma-like molecules were also found in mtDNA preparations of other plant species (20, 38), supporting the view that rolling-circle replication may be a common phenomenon in mitochondria of higher plants.

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