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Steffen Backert · Thomas Börner

Phage T4-like intermediates of DNA replication and recombination in the mitochondria of the higher plant *Chenopodium album* (L.)

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Abstract We have studied intermediates of the recombination and replication of chromosomal mitochondrial (mt) DNA prepared from suspension cultured cells of *Chenopodium album* (L.) by electron microscopy during the whole growth cycle. We identified several types of potential recombination and replication intermediates including rosette-like structures, as well as other branched and sigma-like molecules. The absolute and relative amounts of these structures changed dramatically during the growth cycle, indicating high dynamics in the structural organization of the mt genome. The rosette-like molecules had sizes of 2–5 genome units and were found to contain putative replication forks and 'Holliday'-junctions known from recombination intermediates. The high number of rosettes during the first days of culture, and their drastic reduction in the stationary growth stage, were found to be inversely related to changes in the quantity of linear molecules of 40–200 kb. This observation suggests that linear molecules participate in the formation of giant branched rosette-like structures. Most linear molecules were previously found to have at least one single-stranded end, which may allow recombinative invasion of other double-stranded molecules. Thus, recombination events may lead to the formation of more complex molecules and initiate replication similar to phage T4. We propose the coexistence of a recombination-dependent mode of replication with a presumably recombination-independent rolling-circle mode of replication in the mitochondria of *C. album*.

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S. Backert[1] (✉) · T. Börner
Institut für Biologie, Humboldt-Universität zu Berlin,
Chausseestrasse 117, D-10115 Berlin, Germany
e-mail: backert@mpiib-berlin.mpg.de
Tel.: +49-30-28460420; Fax: +49-30-28460401

Present address:
[1]Max-Planck-Institut für Infektionsbiologie,
Abteilung Molekulare Biologie, Monbijoustrasse 2,
D-10117 Berlin, Germany

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Introduction

Homologous recombination and DNA replication are tightly interrelated processes. Chromosomal or plasmid DNA replication in some organisms can be triggered by recombination processes (Cohen and Clarke 1986; Viret et al. 1991; Kogoma 1997) and recombination can stimulate both sigma- and theta-types of replication (Noirot et al. 1987; Morel-Deville and Ehrlich 1996). The best-studied models of recombination-dependent replication are bacteriophage T4 (Kreuzer and Morrical 1994; Mosig 1998) and two distinct modes of replication in *Escherichia coli* depending on the strand-exchange activity of the RecA protein (Kogoma 1997). Other interesting examples are the unusual genome of *Borrelia* segmented among several linear replicons (Saint Girons et al. 1994) and the mitochondrial (mt) genome from some protists, fungi (including baker's yeast) and possibly higher plants (Bendich 1993; Preiser et al. 1996; Backert et al. 1997c). The mtDNA from higher plants is probably the most extreme example because of its large size (200–2400 kb), complex organization, and rapidly changing structural organization due to recombination events contrasting to its conservatism with respect to point mutations (Rode et al. 1987; Palmer and Herbon 1988; Small et al. 1989; Hanson and Folkerts 1992; Vitart et al. 1992; Kanazawa and Hirai 1994; Muise and Hauswirth 1995; Wolstenholme and Fauron 1995; Unseld et al. 1997; Albert et al. 1998; Janska et al. 1998). Structural models, based on restriction-mapping data, depict either a genome-sized circular chromosome, the master circle, in balance with a limited number of predictable large subgenomic circles (Lonsdale et al. 1984; Palmer and Shields 1984), or only subgenomic chromosomes without a master circle (André and Walbot 1995). Yet, plant mtDNA preparations revealed a much

higher diversity of subgenomic molecules, including heterogenous populations of linear molecules, circles and more complex molecules such as rosette-like structures, as well as plasmids. Their origin, function and relation to the genetic entity of the mt genome are not understood (Backert et al. 1997c).

Very little is known about recombination and replication mechanisms in plant mitochondria. In planta, replication of mtDNA seems not to occur in all cells. In *Pelargonium* roots mtDNA synthesis was detected only in specific cells (Kuroiwa et al. 1992). Investigation of tobacco suspension-cultured cells revealed preferential DNA synthesis in the mitochondria (and plastids) prior to nuclear DNA synthesis just after the stationary phase when cells were transferred to fresh medium (Suzuki et al. 1992). Two short stretches of chromosomal mtDNA of *Petunia hybrida* harboring putative replication origins have been identified by an in vitro assay (de Haas et al. 1991). The origins of circular mt plasmids have been characterized in *Vicia faba* and *Chenopodium album*, initiating unidirectional theta (Wahleithner and Wolstenholme 1988) and rolling-circle (RC) replication (Backert et al. 1996a), respectively. Theta-like replicating mtDNA circles have also been detected in rice (Wang et al. 1989). Oldenburg and Bendich (1996, 1998) investigated replicating mtDNA molecules of tobacco and liverwort by pulsed-field gel electrophoresis (PFGE) and moving pictures under a light microscope. A high percentage of the mtDNA was found as complex forms with multiple DNA fibres. This finding led to the conclusion that replication may be initiated by recombination events that create branched structures. Similar observations have also been made in the mitochondria of *Brassica* (Scissum-Gunn et al. 1998), fungi (Bendich 1996) and the malarial parasite (Preiser et al. 1996). These results suggest replication mechanisms completely different from, or in addition to, that observed for the mtDNA of vertebrates (Schmitt and Clayton 1993), trypanosomatids (Carpenter and Englund 1995) or for certain linear fungal mt chromosomes (Dinouel et al. 1993). Detailed knowledge about the mechanisms of replication and recombination may help to understand the in vivo organization of the mt genomes in higher plants as well as in protists, yeast and other fungi.

Over the last few years we have studied the structure and replication of mtDNA from the dicot *Chenopodium album* and found evidence for a sigma (RC)-type of replication (reviewed in Backert et al. 1997c). Electron micrographs of replication intermediates revealed about 18% of sigma-like molecules among the subgenomic circles during the logarithmic growth phase of suspension-cultured cells. About half of the sigma-like molecules had tails exceeding the length of the corresponding circle suggesting the formation of concatemers similar to bacteriophage λ, λ-type (Bastia and Sueoka 1975), and only a few were of the ϕX174-type (Koths and Dressler 1978) with single-stranded (ss) tails. The RC-type replication cycle of the mt plasmid mp1 (1309 bp) from *C. album* was characterized in vivo (Backert et al. 1996a, b) and in vitro (Backert et al. 1998). The high amount of ss mtDNA, the distribution of ssDNA stretches in the sigma- structures as well as the detection of entirely ssDNA molecules provided further evidence for an RC-type of replication in these organelles (Backert et al. 1996a, 1997a, Backert 2000). However, the detected number and size of sigma-type replication intermediates could hardly account for all expected replication intermediates of the complete genome in the mitochondria of dividing cells and does not provide an explanation for the origin and function of the highly complex rosette-like DNA structures observed in a large number in the mitochondria of *C. album* (Backert et al. 1997a).

To further elucidate the mechanisms of DNA replication and recombination in the mitochondria of *C. album*, we studied changes in the size and structure of DNA molecules during the growth cycle of cells in a suspension culture by EM. By the quantitative evaluation of thousands of EM pictures the mtDNA from *C. album* was found to produce highly branched rosette-like structures preferentially between the 1st and 3rd day of culture. Closer inspection of these and other branched molecules revealed mechanistic similarities to intermediates of alternative recombination-dependent modes of replication in bacteria and bacteriophage T4 (reviewed by Kreuzer and Morrical 1994; Kogoma 1997; Mosig 1998), in which recombination between a free 3′OH end and an internal homologous nucleotide sequence gives rise to a replication fork, thereafter creating networks of branched concatemers.

Materials and methods

Plant material

Suspension culture C.9.1 of *C. album* was cultivated as described (Backert et al. 1996a). Cells were harvested 1, 3, 6, 9 and 12 days after transfer into new medium.

Preparation of mitochondria and mitochondrial DNA for electron microscopy

Mitochondria were isolated from protoplasts, purified by differential centrifugation and treated with DNaseI, followed by centrifugation on a discontinuous sucrose gradient (Backert et al. 1996a). In order to minimize the danger of mechanical breakage of large mtDNA molecules, purified mitochondria were first immobilized in LMP-agarose, lysed, and then washed as described (Backert et al. 1996b). Contaminating RNA was removed by digestion using the ribonuclease cocktail of Stratagene (La Jolla, USA).

LMP-Agarose blocks with embedded mtDNA were digested with gelase following the protocols of the supplier (Biozym, Hameln, Germany). Remaining undigested LMP-agarose was pelleted by centrifugation for 5 min at $2000 \times g$. The supernatant containing mtDNA molecules was directly prepared for EM analyses. To further minimize the danger of mechanical breakage of large mtDNA molecules during preparation, we utilized the gentle droplet method (Lang and Mitani 1970). The droplet solution contained 200 mM of ammonium acetate pH 7, 1 mM of EDTA and, in part of the experiments, 0.001% cytochrome c. Except for the molecule shown in Fig. 2a, all EM micrographs presented in this paper show mtDNA molecules prepared with cytochrome c.

A transmission electron microscope E400T (Philips, Eindhoven, Netherlands) operating at 80 kV was used in all investigations. The sizes of mtDNA molecules were determined by comparison with the length of an internal standard, plasmid pBR322 (4363 bp).

Results

The aim of this study was to follow changes in the structure of mtDNA from *C. album* suspension-cultured cells during the whole growth cycle, and to identify intermediates of the recombination and replication processes. For this purpose, we analyzed DNA molecules from purified mitochondria harvested during a time course of 1 and 3 days (early growth stage), 6 days (logarithmic growth stage) and 9 or 12 days (stationary growth stage) of culture, respectively. The purity of the mtDNA was checked by restriction-enzyme digests and Southern hybridization. Hybridization of isolated mtDNA with a nuclear 18S *rRNA* gene or the plastid *rbc*L gene as a probe did not show contamination by nuclear or plastid DNA (data not shown). To avoid random overlapping of molecules, the mtDNA concentration in our preparations was chosen to be low so that artifacts of this type could be largely excluded. From each preparation, obtained by the very gentle droplet procedure, 400 mtDNA molecules were randomly selected for analysis by quantitative EM (Fig. 1). Two independent mtDNA preparations from each time point gave nearly identical results. All grids contained heterogeneous populations of linear, circular and more-complex molecules. The size range found within each molecular type is displayed in Table 1.

Linear molecules and rosette-like structures

A main fraction of the molecules, up to 80% of the individual molecules analyzed in each fraction, had a simple linear morphology with variable sizes. Most of these molecules fell in the size range of 40–200 kb throughout the growth cycle, though 8 out of 400 molecules measured after 3 days of culture and two molecules measured after 6 days of culture were larger than the *Chenopodium* mt genome unit of approximately

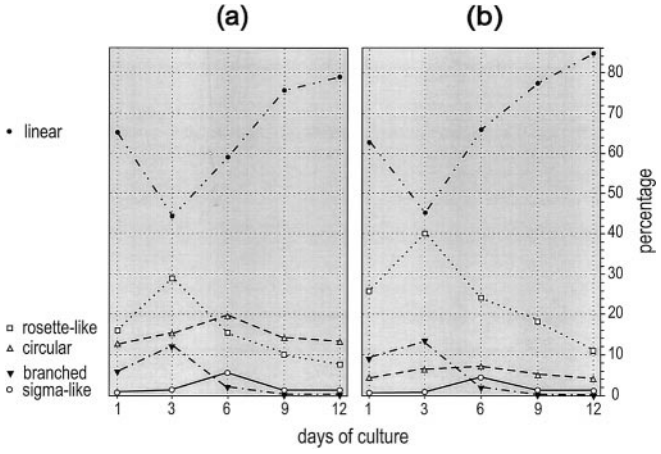


Fig. 1a, b Quantitative electron microscopic analysis of mitochondrial DNA molecules from *C. album* during the whole growth cycle of suspension-cultured cells after the transfer of cells into new medium. For simplification and better comparison the data are presented in percent. **a** shows changes in number of individual molecules and **b** gives the corresponding data on the total size of these molecules (measured in kb). For more details see Table 1

270 kb and had sizes between 290 and 780 kb. The number and sizes of linear molecules were very high 1 day after the transfer of cells into new medium and in the stationary growth phase. The lowest amount (43.7%) of linear molecules was observed after 3 days of culture. At the same time we found the highest number of molecules in the form of very large tangled aggregates (116 rosette-like structures) and 49 linear or circular molecules with one or more branches reminiscent of intermediates of replicating and recombining DNA from bacteriophage T4 or T7 (e.g. Huberman 1968; Powling and Knippers 1976; Masker et al. 1978; Dannenberg and Mosig 1983) or vaccinia or herpes simplex viruses (Dahl and Kates 1970; Severini et al. 1996) and highly supercoiled DNA as shown for protein-free phage P4 DNA (Liu et al. 1981). Many of the extremely large rosette-like structures appeared to have distinct dense areas (hereafter called 'cores'), as previously also observed in mtDNA preparations from other plants (Fontarnau and Hernández-Yago 1982; Dudareva et al. 1988; Wang et al. 1989) or petite yeast (Locker et al. 1979). Usually, the rosettes had the appearance shown in

Table 1 Size distribution of mitochondrial DNA molecules during a growth cycle of suspension-cultured and plant cells from *C. album* (L.)[a]

Shape of molecules		Size distribution of molecules in:					Plants after 3 weeks[b]
		Suspension-cultured cells harvested after					
		1 day	3 days	6 days	9 days	12 days	
Linear	–	1–270	2–780*	1–650	1–200	1–180**	1–240
Circular	○	1.3–140	1.3–140	0.3–183*·**	1.3–43	1.3–56	1–140
Sigma-like	○–	1.3–20	1.3–85	1.3–128*·**	1.3–25	/	1.3–20
Branched	✕	5–120	11–320*·**	10–80	6–56	/	5–20
Rosette-like	❀	4–450	10–1500*·**	4–750	5.5–130	4–86	1.3–140

[a] A total of 400 individual molecules were analyzed in each fraction, sizes given in kilobase pairs (kb); Asterisks indicate the largest sizes* and number** of molecules of the respective type
[b] Data taken from Backert et al. 1996b

Figs. 2b and 3a, b. Omitting cytochrome c in the droplet preparation mixture, as suggested by Leon and Macaya (1983) to avoid artifacts, did not produce different pictures of the rosettes except for a reduction in the thickness of the cores (compare arrows in the rosettes of Fig. 2a, no cytochrome c, and Fig. 3a, with cytochrome c). The large concentration and partial aggregation of the mtDNA in the interior of the complex prevented any conclusion on the secondary or tertiary structure of the DNA in these regions. However, most of the DNA fibres emerging from the aggregates were sufficiently untangled to allow length measurements. The overall size and DNA amount of the rosettes were the highest after 3 days of cell culture just before the logarithmic growth stage when multiple cell division starts. At this time point, about 30% of the rosettes were larger than the mt genome size. The maximum length measured was about 1500 kb, or more than five genome units (Fig. 3a). Our earlier attempts to determine the size and structure of intact rosette-like mtDNA by PFGE after digestion with rare cutting restriction enzymes led to the conclusion that mainly very long molecules form these structures because fragments up to 170 kb could be obtained as single bands (Backert et al. 1997a). Since the

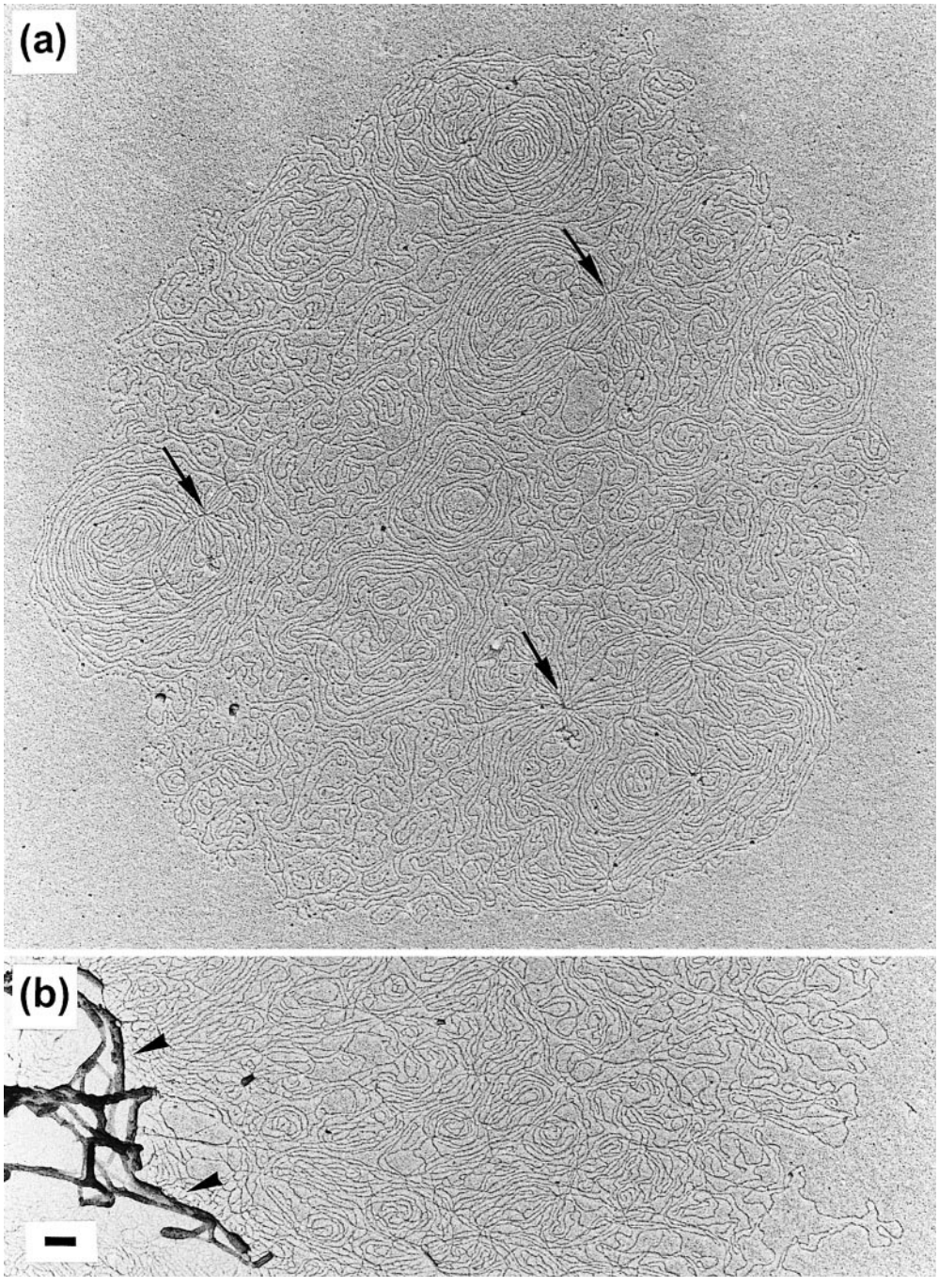


Fig. 2a, b Electron micrographs of giant mitochondrial rosette-like structures from *C. album*. **a** shows a structure of about 1300 kb having no distinct "cores", but several regions where many DNA fibres emanate from particular points. *Arrows* indicate three examples of such regions. **b** Section of a complex rosette structure having a scaffold-like dense center ("core", see *arrowheads*) with many DNA fibres attached (bar represents 1 kb)

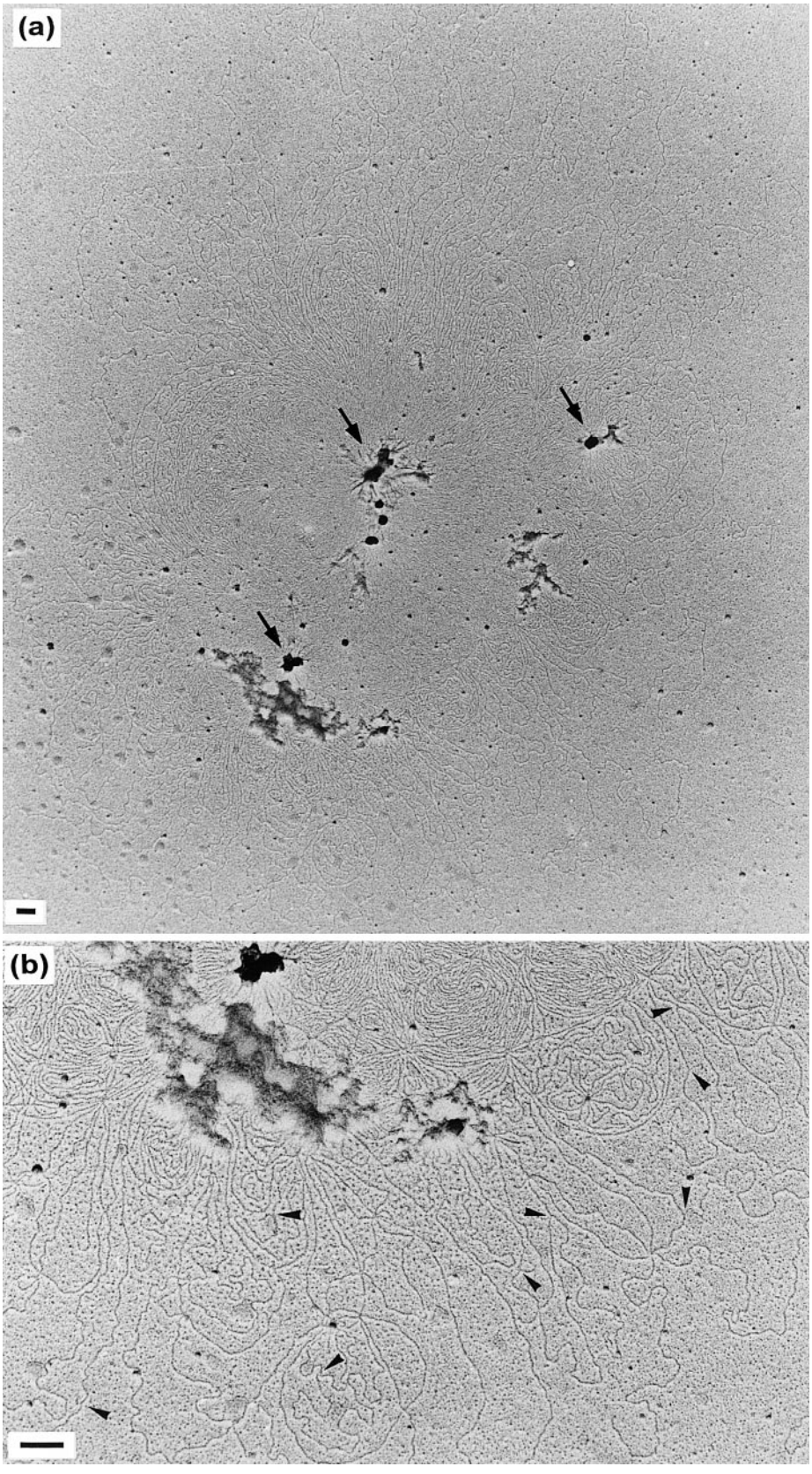


Fig. 3a, b A highly branched mitochondrial rosette-like structure from *C. album*. **a** this large complex is about 1500 kb in size and bears a few distinct dense "core" particles. Three examples of cores are indicated by *arrows*. **b** *arrowheads* in this enlarged section of the lower part of **a** indicate forks (bars represent 1 kb)

rosette-complexes survive the normal mt lysis procedure with 1 mg/ml of proteinase K and 1% SDS, it seems unlikely that they are held together by proteinaceous material although in some cases the morphology of the core particles was similar to remnants of nucleoid (or scaffold-like) or membrane-bound DNA complexes (for

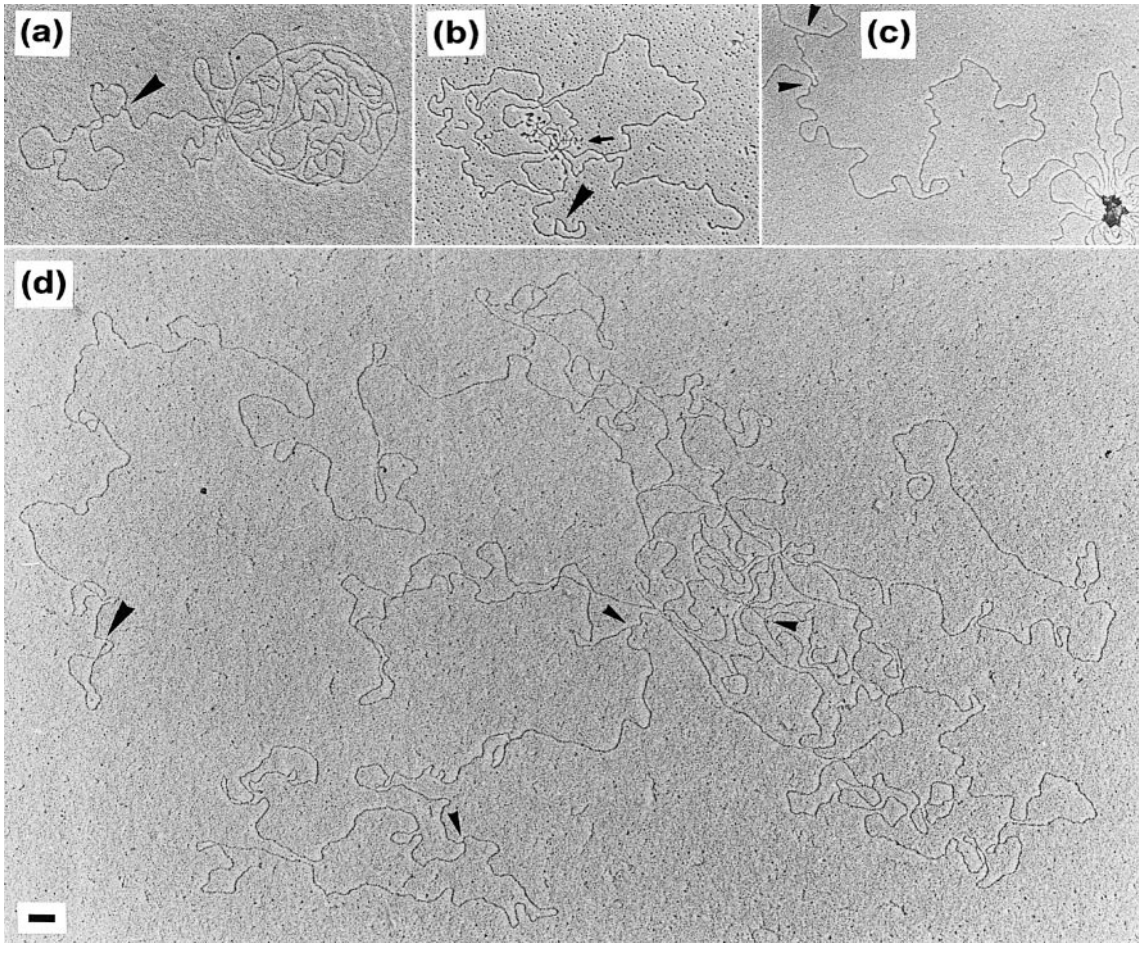
example see arrowheads in Fig. 2b). As a control, pBR322 circular marker molecules rarely produced rosettes. Such rosettes were exclusively of monomer size and did not form large complexes. Attempts to break mtDNA rosettes by a more extensive pre-treatment with proteinase K or by spreading on a hyperphase containing 40% formamide were unsuccessful (Backert et al. 1996b). On the other hand, the rosette-complexes were completely digested by treatment with DNaseI (Backert et al. 1997a). Therefore, the possibility exists that the rosettes are held together by DNA junctions. Indeed, at higher magnification we observed fork-like structures in the rosettes with a more loose appearance (see arrowheads in Fig. 3b). Several rosettes clearly showed also the presence of putative recombination/replication forks (examples in Fig. 4a–d) and stretches of collapsed ssDNA (Fig. 4b). Forks are likely to occur with much higher frequencies in the rosettes than could be detected due to poor spreading of most of these structures.

Fig. 4a–d Detection of branched regions in rosette-like DNA molecules from *C. album* mitochondria. Four spread rosette structures having internal forks (*small arrowheads*) and/or linear DNA fibres with a loop at the end (*large arrowheads*) are shown (**a**–**d**). The *arrow* in **b** indicates a large stretch of collapsed single-stranded DNA (bar represents 1 kb)

Circular and branched molecules

The number of circular mtDNA molecules was relatively low over the whole growth cycle. Their percentage ranged between 5 and 20% with a small peak in the logarithmic growth stage. The circles had contour lengths ranging from 0.5 to 183 kb, but the main fraction (>80%) had variable sizes between 1 and 25 kb. Consequently, the DNA amount (measured in kb) of circular molecules in the total fraction of mtDNA is very low (Fig. 1b). 1.5–2.5% of the circles were found in catenated form. Throughout the growth cycle, about one-half of the circles were represented by the 1.3-kb plasmid mp1, which is in agreement with earlier observations (Backert et al. 1996a, b).

Altogether 27 circles with one linear tail were counted as a separate class of sigma-structures or rolling-circles (RCs). Examples are depicted in Fig. 5b, c. The number of the RCs was highest in the logarithmic growth stage (6 days after cell transfer), as in our previous studies. About 40% of the sigma-structures had tails longer (up to 40-fold) than the circumference of the respective circles, suggesting the formation of concatemers during RC replication. Furthermore, we detected two circles with a branched tail (Fig. 5d), three structures in which circles were interconnected with linear tails (Fig. 5e) and four



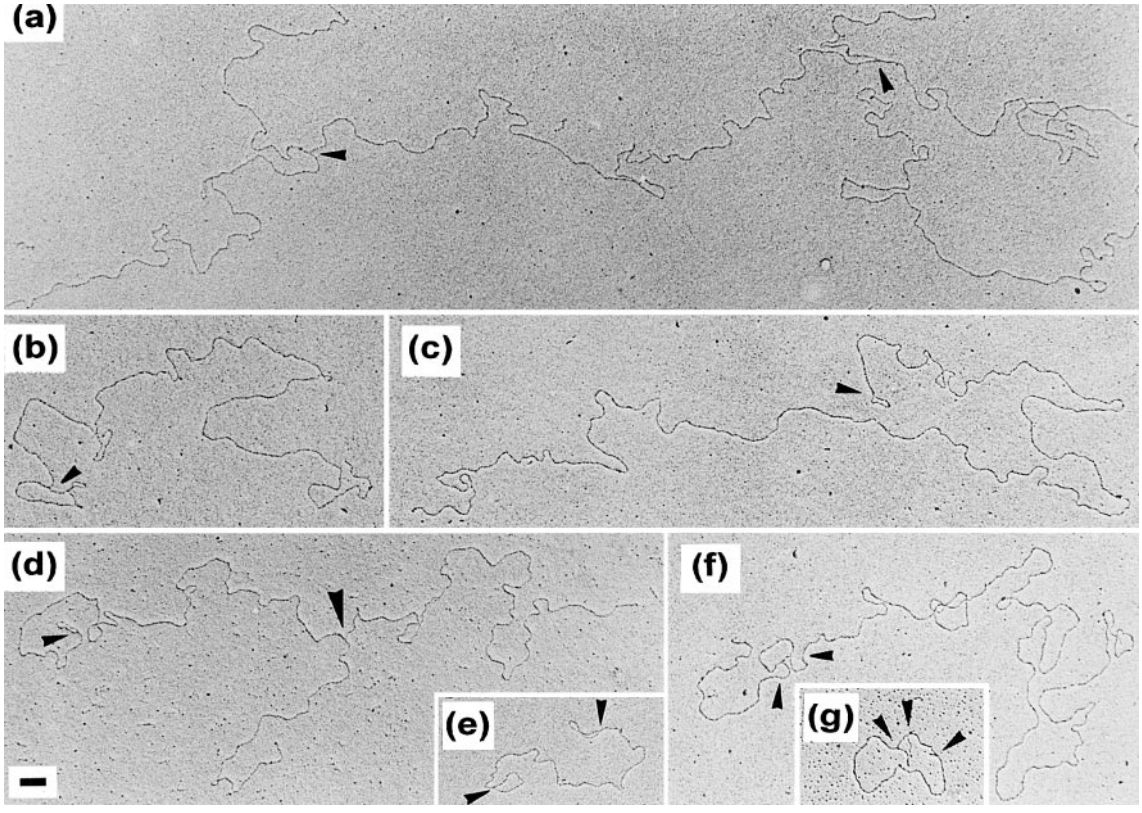


Fig. 5a–g Branched linear and circular molecules observed in chromosomal DNA from *C. album* mitochondria. **a** section of a very large linear mitochondrial DNA molecule having two branches. **b** circular molecule of 5 kb with a linear tail of 25 kb. **c** circular molecule of 1 kb with a linear tail of 40 kb. **d** circular molecule of 1 kb with a branched linear tail of 40 kb. **e** two circles (2.5 kb each) interconnected by a linear tail of 13 kb. **f** circle of 3 kb with two linear tails (6 kb and 30 kb). **g** circular molecule (10 kb) with three linear tails of 1 kb, 1.3 kb and 0.5 kb, respectively. *Arrowheads* indicate fork regions (bar represents 1 kb)

sigma-molecules forming a loop within the circle (Figs. 6a, b).

Less complex branched structures formed another group of 68 molecules. We cannot rule out that part of these molecules resulted from the breakage of larger structures during preparation. The branched molecules (except the simple sigma-structures) grouped together had a peak in number and size just after 3 days culture (compare Fig. 1, Table 1). Among them only a very few (three) circular and linear molecules included bubbles with two identical arms, as typical for theta-type replication. The main fraction of this class included linear molecules with one branch (unequal simple Ys) and linear molecules with two (unequal H-structures) or more branches (Fig. 5a). Additionally, unequal X-forms (Fig. 7) and circles with two (Fig. 5f) or three (Fig. 5g) linear branches were observed. Most of these molecules can be possibly interpreted as recombining and replicating structures. A potential example of a circle apparently disintegrating by recombination from a much longer linear molecule or invasion/annealing of a linear molecule is shown in Fig. 7a. This and other molecules

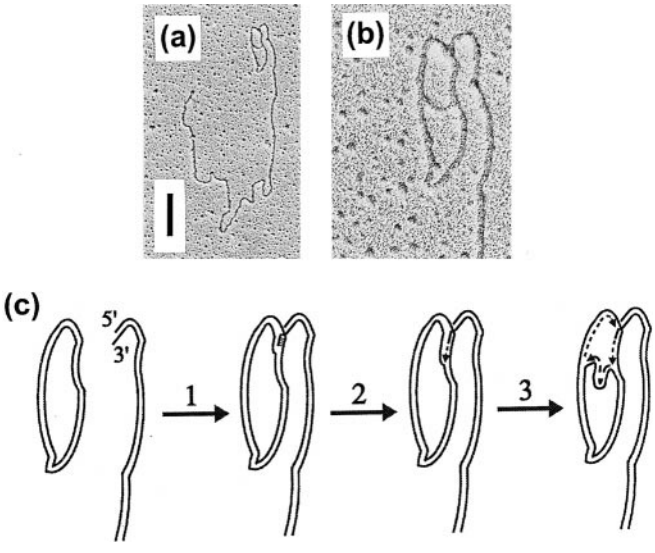


Fig. 6a–c Electron micrograph of a circular molecule of 2.5 kb having an internal displacement-loop and a linear tail of 10 kb prepared from *C. album* mitochondria (**a**). Magnification of the circle-tail junction (**b**). A model to explain the origin of the structure shown in **a** by a recombination-dependent type of replication involving a circle invaded by a linear molecule with a ss end (**c**). In a first step, a linear molecule having a free 3′OH end may pair with a homologous region of the circle (as known from RecA-dependent recombination). Only an invasive 3′OH end can be utilized as a primer for replication in the indicated direction. The formed D-loop structure could be recognized, and replication be started, by a D-loop-type primosome with the subsequent loading of a replication fork helicase, DNA primase and the DNA polymerase (see Kogoma 1997; Cox 1998) (bar represents 1 kb)

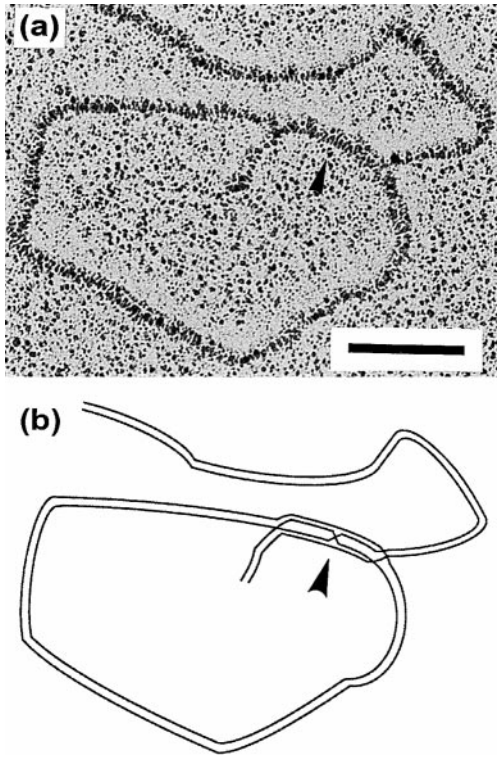


Fig. 7a, b Potential recombination junction in a chromosomal DNA molecule from *C. album* mitochondria at high magnification. Arrowheads indicate the putative position of strand exchange in one or more recombining molecules (**a**). A model shows one possible explanation of the structure shown in **a** by a circle recombining out of a linear molecule via crossing over in a potential 'Holliday'-junction (**b**). (bar represents 0.5 kb)

showed putative cross-over structures as known from a 'Holliday'-junction (Holliday 1964) reminiscent of *recA*-directed recombination junctions described for bacteria (reviewed by Lloyd and Sharples 1992; Stahl 1994; Eggleston and West 1996; Kogoma 1997; Cox 1998).

Discussion

Investigations on the replication of plant mtDNA are mainly hampered by the highly complex structure of this genome and the non-existence of a suitable transformation system for plant mitochondria. Consequently, only a few details are known so far about the mode or origins of DNA replication and virtually nothing about recombination in these organelles, although a high rate of recombination is expected to occur because of the presence of many repeated sequences in the mtDNA and the observation of frequent rearrangements (Small et al. 1989; Hanson and Folkerts 1992; Wolstenholme and Fauron 1995; Unseld et al. 1997; Albert et al. 1998; Janska et al. 1998). We have been studying organization and replication in cells of a suspension culture of mtDNA of *C. album* because it is evident that replication has to occur during the cell cycle. In previous studies we were able to demonstrate that a small plasmid, called mp1, replicates via a sigma-type of intermediate in *C. album* mitochondria. Also larger subgenomic circles seem to use the same RC-type of replication (Backert et al. 1996a; Backert 2000). However, the sigma-like structures formed only a relatively small fraction among the different types of mtDNA molecules. The only other observed candidates for replication (and/or recombination) intermediates would be the more complex structures represented mainly by the rosettes. Large, branched molecules were also observed in mtDNA preparations from liverwort and tobacco analyzed under a light microscope and regarded as intermediates of a phage T4-like type of replication (Oldenburg and Bendich 1996, 1998).

In the present study, we conducted an extensive EM investigation of all types of chromosomal mtDNA molecules (excluding plasmid mp1) from *C. album* with a relatively small genome size of 270 kb and followed changes in their molecular structure throughout the growth cycle. We made the following observations which may deepen our understanding of the structure and replication of plant mtDNA.

(1) Although the various types of molecules were found during all stages of the growth cycle, we observed the highest number of complex molecules early after transfer of the cells into new medium, whereas the highest number of circles including sigma-like molecules and of linear molecules appeared during the phase of logarithmic growth and in the stationary phase, respectively. These data indicate a dynamic in the structural organization of the mt genome and, moreover, because of its reproducibility, strongly suggest that the observed complex molecules occur in organello and thus are not artifacts of preparation. This view is further supported by previous observations. We detected rosettes with and without cores only in the well-bound fraction and not in other mtDNA fractions after separation by PFGE (Backert et al. 1997a). Of course, the true in situ organization of the observed complex structures cannot be deduced from EM pictures since, in organello, proteins are bound to DNA and the DNA may be bound to membranes. 'Core' structures within rosettes were interpreted as artifacts caused by attachment of DNA to cytochrome c during the preparation for EM at ammonium acetate concentrations lower than 150 mM (Leon and Macaya 1983). Therefore, we have used 200-mM ammonium acetate and prepared mtDNA with or without the addition of cytochrome c. No significant differences in the shape of the rosettes except for the thickness of their cores have been detected (compare Figs. 2a and 3a). Previously, we also observed similar structures in preparations without cytochrome c using the mica adsorption method (Backert et al. 1997a). Thus, the observed cores may indeed exist within the rosettes in vivo.

(2) Within the rosettes, comprising up to 2–5 mt genome units, we could detect branched regions resembling replication forks as well as structures which are best interpreted as 'Holliday'-junctions (Holliday 1964).

This observation supports the idea that the large rosette-like DNA molecules represent intermediates of replication and recombination.

(3) In parallel with the increase in number and sizes of the rosettes, the number of linear molecules decreases during the first days of culture whereas we observed the opposite situation in the stationary phase. This phenomenon would find a plausible explanation if the complex structures seen as rosettes in EM pictures were formed mainly from linear molecules during the early growth stage and were later processed to give rise again to linear molecules, supporting the model of a precursor-product relationship between more complex and more simple types of mtDNA molecules (Oldenburg and Bendich 1996). Both the formation and processing of rosettes could involve homologous and/or site-specific intermolecular and intramolecular recombination events (Fig. 8).

We can only speculate on how recombination and replication may proceed within the large branched molecules. Our results suggest that *Chenopodium* mitochondria possess highly complex intermediates of recombination-dependent replication, the rosette-like molecules, in addition to the sigma-like intermediates of RC replication. The main molecular changes undergone by mtDNA molecules during the growth of cultures of *Chenopodium* cells seem to involve recombination, thereby producing the complex rosettes with a branched nature at the expense of linear molecules. At least part of the rosettes may represent concatemers, as many of them are much larger than genome size. In current models of homologous recombination, the process is thought to be initiated with the invasion of a duplex DNA molecule by a ssDNA or a ss end of a dsDNA molecule (e.g. Lloyd and Sharples 1992; Stahl 1994; Kogoma 1997).

In *E. coli* cells dsDNA molecules are recognized and processed by the RecBCD enzyme complex to yield invasive ssDNA with free 3′OH ends (e.g. Eggelston and West 1996). This structure develops into a 'Holliday'-junction by a process involving RecA and branch migration which then leads to the integration of a foreign fragment into a recipient DNA molecule. That comparable processes might occur in plant mitochondria is supported by our observations. The observed forks and putative Holliday junctions in rosette-like structures are an indication of temporally ongoing replication and recombination (Figs. 3 and 4). These observations support the view of the occurrence of a process in plant mitochondria similar to an alternative mechanism of bacterial DNA replication (Kogoma 1997) or recombinational repair called replicative homologous recombination (Cox 1998; Kanaar et al. 1998). Circular molecules represented only minor fractions of the mtDNA molecules. These molecules may arise spontanously by recombination between repeats within the rosettes or other molecules, as proposed in Fig. 7.

In a previous study we detected a strikingly high proportion of ssDNA in the mitochondria of *Chenopodium* (Backert et al. 1997a). By attachment of the *E. coli* SSB protein to mtDNA, free ss stretches were found at one or both ends of the majority of linear molecules thus providing one precondition for recombination, potentially invasive linear molecules. It seems plausible that ss ends of linear molecules can pair with homologous regions in other molecules in *Chenopodium* mitochondria, as also found in yeast (Williamson 1975; Sena et al. 1986; Lockshon et al. 1995) or microbial (Preiser et al. 1996) mitochondria. The recombinative invasion of a linear molecule into a ds template would result in a D-loop, from which DNA replication can be initiated (e.g. Luder and Mosig 1982; Formosa and Alberts 1986; Viret et al. 1991; Kreuzer and Morrical 1994; Kogoma 1997). Similarly telomer loops at the ends of linear mammalian chromosomes formed by invasions might trigger DNA replication (Griffiths et al. 1999). The same

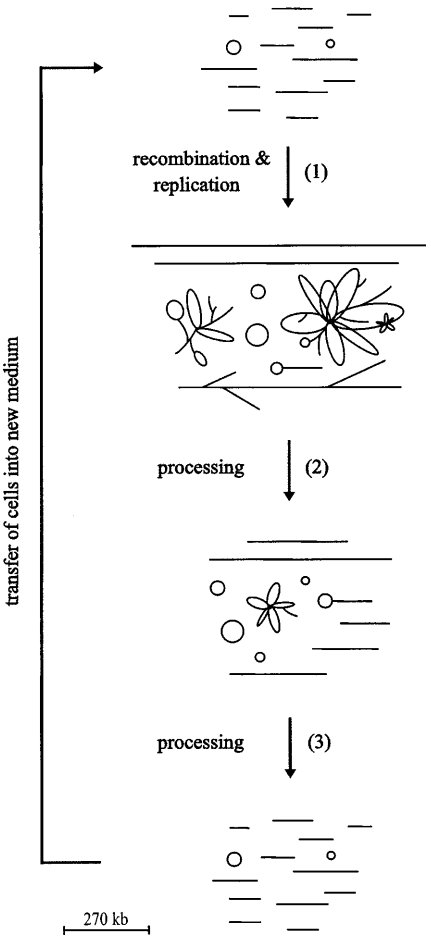


Fig. 8 Simplified hypothetical model for structural changes of mitochondrial DNA molecules during a growth cycle of *C. album* suspension cultured cells as observed by electron microscopy. *1* during early stages of growth, mitochondrial DNA recombination and replication seem to be tightly interrelated thereby producing large branched concatemers (rosette-like molecules) with sizes up to 2–5 mitochondrial genome units. *2* rolling-circle replication of subgenomic circles and processing events of the concatemers occur predominantly during the logarithmic growth stage. *3* final processing of concatemers mainly to 40–200 kb-long linear molecules was found when cells entered the stationary growth phase. The *bar* represents a mitochondrial genome size of about 270 kb. For more details see text.

mechanism would apply to any repetitive DNA at the ends (also for the mtDNA of *C. album*), regardless of how repetitions are generated. An RNA-polymerase-dependent initiation via R-loops is known from alternative bacterial DNA replication and early DNA replication of phage T4 (Luder and Mosig 1982; Kogoma 1997). We also observed potential recombination/replication intermediates in which a linear molecule invades a circle, followed by replication (Fig. 6). Moreover, this study revealed the existence of circles with two or more tails (Fig. 5f, g), linear molecules with one or more branches (Fig. 5a) and circles with branched tails (Fig. 5d), suggesting the presence of multiply branched sigma-type replicating linear molecules and/or multiple linear molecules invading circular or linear templates. The latter recombination-dependent mechanism was found to occur early after the onset of origin-dependent (RNA-polymerase dependent) replication or in a different pathway that occurs late (Kreuzer and Morrical 1994; Mosig 1998).

Taken together, our results support the hypothesis that a recombination-dependent mechanism of replication (with features known from bacteria and, in particular, from phage T4 replication) coexists with a λ- and ϕ X174-type of RC replication in *Chenopodium* mitochondria. The use of more than one type of replication in the mitochondria of one and the same species seems to be not unusual (Fangman et al. 1990; Maleszka et al. 1991; Bendich 1993; Rayko and Goursot 1996; Backert et al. 1997c; Nosek et al. 1998). Further studies on enzymes involved in recombination and replication in plant mitochondria, the development of in vitro replication systems as well as the identification of replication origins and the characterization of the ssDNA regions, are urgently needed to understand the interplay of recombination and replication in the structure and function of plant mt genomes.

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