真正粘菌のミトコンドリアの融合とゲノムの再構成を誘起するミトコンドリアプラスミドの解析

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Structural features and genetic organization of the mF plasmid that promotes mitochondrial fusion and reconstruction of mitochondrial genome in *Physarum polycephalum*

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SUMMARY

The mitochondria of *Physarum polycephalum* have a linear plasmid (mF) which promotes a mitochondrial fusion. Moreover, the restriction fragment length polymorphism (RFLP) analyses have suggested that the reconstruction of the mitochondrial DNA (mtDNA) occurs with the mitochondrial fusion promoted by this plasmid in zygote. In this study, to analyze the phenomena associated with mitochondrial fusion at the molecular level, I have done experiments as follows.

In CHAPTER I, the structural features of the mF plasmid have been determined. It was a linear molecule with three terminal inverted repeats (TIRs) at each end. The most characteristic feature was a 144-bp repeating unit which existed between a 205-bp TIR at the extreme ends of the mF plasmid and another 591-bp TIR. Moreover, in the right terminal region, there was an open reading frame (ORF) which had extensive homology with polymerization domain of the DNA polymerases.

In CHAPTER II, the reconstruction of the mitochondrial genome, which occurs during plasmodium formation, has been analyzed at the molecular level. In matings between mF^+ and mF^- strains which respectively carry and do not carry the mF plasmid, recombination occurred between the mtDNA and the mF plasmid, and recombinant mtDNA with the ends of the mF plasmid as its ends was generated. The sequence study revealed that the mF plasmid had a 475-bp sequence that was identical to, but very slightly shorter than, a 479-bp sequence of the mtDNA. The recombination was due to reciprocal crossing-over at this identical sequence. It showed that the mF plasmid caused not only the mitochondrial fusion but also the reconstruction of the mitochondrial genome.

In CHAPTER III, genetic organization of the mF plasmid has been analyzed. The nucleotide sequence of the mF plasmid was 14503 bp in size, and contained 10 ORFs. A brief transcription map of the mF plasmid suggested that the transcription initiation site located near the inner end of the TIRs. There were major transcripts of 1.0, 3.4 and 4.6 knt on the upstream region of the transcription map, and these transcripts was about 500 times more in quantity than that of the lower region. These results showed that the mF plasmid was genetically functional.

In CHAPTER IV, to detect gene(s) associated with the mitochondrial fusion, the mitochondrial genomes of the the mitochondrial fusion-deficient (Δmif^-) strains which were isolated from the mitochondrial fusion strains have been analyzed. The organization of the mitochondrial genome in one of these Δmif^- strains showed that the 2.2-kbp region including the identical sequence with the mtDNA was deleted. Moreover, the abundance of the mtRNA decreased to the same value as that of the strains which have no mitochondrial plasmid. These results suggested that the deleted region was important not only for the mitochondrial fusion but also for the transcription of the mF plasmid.

CONTENTS

PREFACE

Mitochondria are energy-producing organelles in eukaryotic cells. The giant mitochondria have been observed with the electron microscopy in many organisms such as human liver cells (Bang and Bang 1957), *Chlamydomonas reinhardtii* (Osafune et al. 1972), *Euglena gracilis* (Pellegrini 1980) and so on. Hoffman and Avers (1973) revealed only one huge and branched mitochondrion per a cell using the three-dimensional model constructed from serial sections of an entire yeast cell. In 1977, by the studies on the computer-aided three-dimensional reconstructions of serial thin sections of 35 entire yeast cells, Stevens (1977) have suggested that the yeast mitochondria undergo a cyclic process of fragmentation and fusion during growth.

On the other hand, from genetic studies with drug-resistant mitochondrial markers, recombination between mitochondrial genes has been demonstrated (Thomas and Wilkie 1968). By considerable knowledge of mitochondrial genetics in the yeast (see review by Dujon 1981), Dujon *et al.* (1974) proposed a general model for recombination and segregation of mitochondrial genes.

At that time, no one has been observed the fusion of the mitochondrial nuclei which is directly associated with the recombination of mitochondrial DNA (mtDNA). The bulk of the mtDNA is packed together with RNA and proteins to form an electron-dense mitochondrial nucleus (Kuroiwa 1982). Using the DNA-binding fluorescent dye, 4', 6-diamidino-2-phenylindole (DAPI), yeast mitochondrial nucleus has been visualized at the light microscopic level (Williamson and Fennell 1975). With this dye, configuration changes of mitochondria have been examined (Sando *et al.* 1981; Miyakawa *et al.* 1984). In the cell of yeast, most of

the small spherical mitochondria fused to form one large, long mitochondrion before cell budding during the cellular G1 period. After fusion of the mitochondria, many mitochondrial nuclei also fused to form one large mitochondrial nucleus. The long mitochondrion was fragmented into many small mitochondria soon after the cell division (Sando *et al.* 1981; Miyakawa *et al.* 1984). Each small mitochondrion contained one small spherical mitochondrial nucleus. Similar fusion and division of mitochondrial nuclei have been observed in the yeast cell during meiosis (Sando *et al.* 1981; Miyakawa *et al.* 1984), suggesting that the recombination of mitochondrial genomes may occur during meiosis. On the basis of these studies, Kuroiwa (1982) proposed that mitochondria perform "mitochondrial meiosis" during cell nuclear meiosis.

In the true slime mould, *Physarum polycephalum*, the mitochondrial fusion has been observed (Nishibayashi *et al.* 1987). Since the mitochondria of *P. polycephalum* contain about ten times more mtDNA than do mitochondria from other sources, the mt-nucleus has facilitated observations of the processes of mitochondrial-nuclear division via the monitoring of the behaviour of the mtDNA by light and electron microscopy (Kuroiwa *et al.* 1977; Kuroiwa 1982; Kawano *et al.* 1983, 1985). The fusion of mitochondrial nuclei during the sporulation stage has been observed in the plasmodial strain Ng by DAPI fluorescence microscopy (Fig. 1; Kawano *et al.* 1991a). These fusions result in the formation of multinuclear mitochondria which are apparently retained in the mature spores. By the fluorescence microscopic studies in spores of many laboratory strains, the mitochondrial-fusion deficient strain was isolated in addition to this mitochondrial fusion strain (Fig. 1; Kawano *et al.* 1991a). The mitochondrial fusion is observed to occur at high

frequency at two stages of the life cycle, during plasmodial formation and during sporulation, in plasmodial strain Ng and in most of its derivatives. On the other hand, the other strains show low frequencies of multinucleate mitochondria at all stages of the life cycle (Fig. 2; Kawano *et al.* 1991a). These strains in *P. polycephalum* have made possible to study the genetic system controlling mitochondrial fusion and the relationship among the mitochondria, and their nuclear fusion and the recombination of their mtDNA.

The genetical analyses with these strains have shown that the mitochondrial fusion depends upon the presence of the single factor (mif^+) which is transmitted preferentially to the progeny, suggesting mif^+ is mitochondrial (Takano 1990; Kawano *et al.* 1991a). The analyses of the mitochondrial genome from these strains have shown that in all mif^+ strains the mitochondrial plasmid of about 16 kbp present and suggested that mif^+ character is carried on this plasmid, named mF (Takano 1990; Kawano *et al.* 1991a). By analyzing restriction fragment length polymorphisms (RFLPs), in mating between mF^- strains that do not carry the mF plasmid, the uniparental inheritance occurs (Kawano *et al.* 1987). In contrast, a mating between mF^- strain and mF^+ strain that carry the mF plasmid may result a reconstruction of the mitochondrial genome with mitochondrial fusion (Fig. 3; Takano 1990; Kawano *et al.* 1991a).

In this thesis, I have analyzed various phenomena which are associated with the mitochondrial fusion and the reconstruction of the mitochondrial genome. The purposes of the present study are as follows. (1) To establish the basis for further study, the structural features of the mF plasmid from the mif^+ strain were determined with the molecular biological techniques. (2) Following these studies, to demonstrate how changes occur with the mitochondrial fusion, the reconstructed mitochondrial genome of the plasmodia resulted from matings between

mF^- and mF^+ strains was analyzed at the molecular level. (3) To define the genetic organization of the mF plasmid, the total sequence of the mF plasmid and the mode of transcription were cleared. (4) To search for the gene(s) associated with the mitochondrial fusion, the mitochondrial genomes of the mitochondrial fusion-deficient strains which were isolated from the mif^+ strains were investigated.

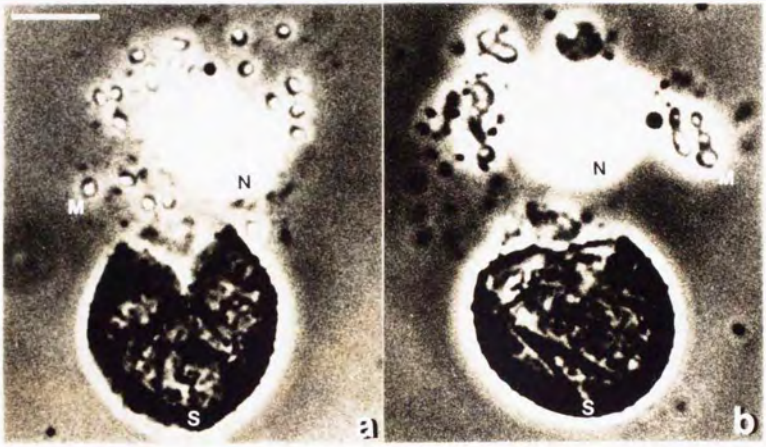

Fig. 1
Spores were cracked open to show the mitochondrial fusion-deficient (a) and mitochondrial fusion (b) phenotypes. There are phase-contrast DAPI-fluorescence micrographs. The mitochondrial nuclei were visible as intense blue spots in the mitochondria as a result of staining with DAPI. M, mitochondrion; N, nucleus; S, spore wall; bar, 5 μm.

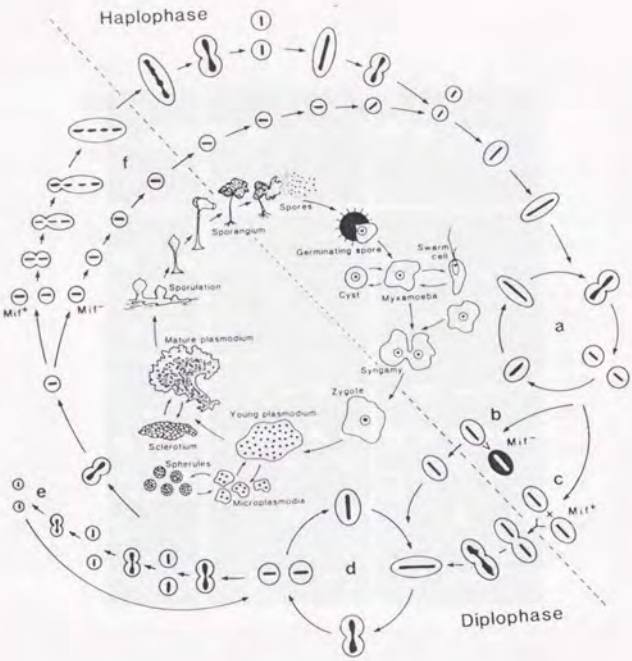

Fig. 2
The life cycle of mitochondria (outer scheme) throughout the life history of *P. polycephalum* (inner scheme). (a) Mitochondrial division cycle in the myxamoeba; (b) hierarchical transmission of mtDNA during formation of plasmodium by crossing between the mitochondrial fusion-deficient strains; (c) mitochondrial fusion promoted by the plasmid during formation of plasmodium by crossing between mitochondrial fusion and fusion-deficient strains; (d) mitochondrial division cycle in the plasmodium; (e) step-wise diminution of mitochondria by the division of mitochondria without DNA synthesis during spherulation; (f) behavior of mitochondria during sporulation. Small spherical, oval and dumbbell-shaped figures in the outer scheme represent mitochondrial bodies and mitochondrial nuclei.

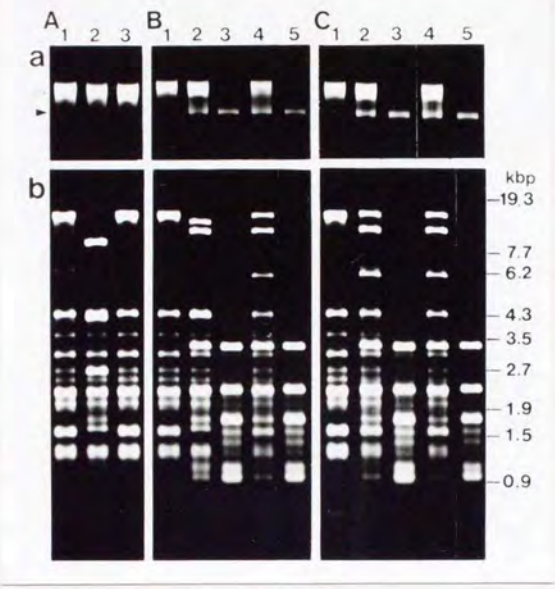

Fig. 3
Agarose-gel electrophoresis of mtDNA showing transmission patterns of mtDNA in matings between myxamoebae. a) Undigested mtDNA (open and closed arrow heads show the bands of mtDNA and the mF plasmid, respectively), b) mtDNA digested with *Hin*dIII. A) The mtDNAs of myxamoebal strains *1* CH934 (mif$^-$), *2* NG6 (mif$^-$), and *3* the product of mating, plasmodium CH934 x NG6 (mif$^-$); B) myxamoebal strains *1* CH934 (mif$^-$), *2* NG7 (mif$^+$), and *4* plasmodium CH934 x NG7(mif$^+$) and the isolated mF plasmid from *3* NG7 and from *5* CH934 x NG7; C) myxamoebal strains *1* CH934 (mif$^-$), *2* OZ35 (progeny of CH934 x NG7; mif$^+$) and *4* plasmodium CH934 x OZ35 (mif$^+$) and the isolated mF plasmid from *3* OZ35 and from *5* CH934 x OZ35.

CHAPTER I

Structural characterization of the mF plasmid which promotes mitochondrial fusion

SUMMARY

The mitochondria of *Physarum polycephalum* have a linear plasmid (mF) which promotes mitochondrial fusion. The restriction map of the mF plasmid showed that it was a linear molecule. Southern hybridization with the ends of the mF plasmid as probes revealed that the mF plasmid included repeating units at both ends. The most extensive array of repeats consisted of at least 17 repetitions of the repeating unit. From the extent of the repetitions, the size of the mF plasmid was estimated to vary from 13.3 kbp to more than 18.3 kbp.

To determine the terminal structure of the mF plasmid, restriction fragments derived from its ends were sequenced. The sequences have shown that the mF plasmid had three terminal inverted repeats (TIRs). The most characteristic feature was a 144-bp repeating unit which existed between a 205-bp TIR at the extreme ends of the mF plasmid and another 591-bp TIR. Moreover, in the right terminal region of the mF plasmid, there was an ORF which covered the entire 591-bp TIR and most of one of the 144-bp repeating units. This ORF encoded a 547-amino acid polypeptide, ORF-547, and showed extensive homology with the polymerization domain of the putative DNA polymerases of linear mitochondrial plasmids from other sources.

INTRODUCTION

Linear plasmids in eukaryotes have been identified in numerous fungi, protozoa and plants, and many linear plasmids appear to be localized in the mitochondria (for review, see Meinhardt *et al.* 1990). The main characteristic feature of these linear mitochondrial plasmids is a terminal inverted repeat (TIR) sequence with a terminal protein covalently linked to the 5' end of the plasmid. A similar terminal structure has been found in adenovirus (Challberg *et al.* 1980) and in bacteriophage φ29 of *Bacillus subtilis* (Gutierrez *et al.* 1988). In these linear DNA viruses, TIRs and terminal proteins are important for the recognition of the origin of replication by DNA polymerase or by DNA-binding protein(s), the 3'-hydroxyl priming of DNA polymerase and subsequent strand displacement during replication of the DNA (Campbell 1986). Analogously, the TIRs and the terminal proteins on the linear mitochondrial plasmids also function in replication (Samac and Leong 1989, Meinhardt *et al.* 1986).

In *P. polycephalum*, the Ng strain and its derivatives contain a linear mitochondrial plasmid (mF) which is apparently responsible for the promotion of mitochondrial fusion (Kawano *et al.* 1991a). Only in strains carrying the mF plasmid, small spherical mitochondria fused with one another to form large multinucleate mitochondria.

To determine the structure of the mF plasmid, the restriction map of this plasmid was constructed. It was a linear molecule and had unique structures at its ends. Therefore, I have sequenced the ends of the mF plasmid. Sequencing studies showed that the mF plasmid had three kinds of TIRs and a putative DNA polymerase that encoded in this region.

MATERIALS AND METHODS

The amoebal strains of *P. polycephalum*, CH934 and NG7, are derivatives of Colonia and Ng, respectively (Youngman *et al.* 1981; Kawano *et al.* 1991a, 1993). Media and growth conditions were identical to those described by Kawano *et al.* (1987).

The mitochondrial plasmid was isolated by the method of Kawano *et al.* (1991a). After complete digestions of the mF plasmid with appropriate restriction endonucleases, in accordance with the manufacturer's instructions (Takara Shuzo Co. Ltd, Japan), the digested fragments were directly cloned into the respective restriction sites of the pBluescript II SK+ vector (Stratagene CA, USA) by the in-vitro cloning technique described by Maniatis *et al.* (1982). Construction of the restriction map was performed as described previously (Takano *et al.* 1990).

The treatment with exonuclease *Bal*31, agarose-gel electrophoresis and Southern blotting were performed as described previously (Takano *et al.* 1990). The treatment with λ exonuclease and Exonuclease III were performed as described by Düvell *et al.* (1988). Southern hybridization with the ECL gene-detection system was performed according to the instructions of the manufacturer (Amersham International Plc, UK).

The terminal fragments of the mF plasmid of the plasmodial strain CH934 x Ng7 were cloned as follows. The total DNA from the isolated mitochondria was treated with T4 DNA polymerase to blunt the ends, and then digested with *Xba*I. The sample was then subjected to electrophoresis. Restriction fragments of approximately 2.0 kbp to 2.5 kbp were purified from the agarose gel and cloned into the *Xba*I-*Sma*I site of the pBluescript II vector. The clones NPT4x-34 and -64 were chosen for determination of the DNA sequences of the left and right ends

of the mF plasmid, respectively. These fragments were recloned into the pBluescript II SK- vector for determination of the DNA sequences of the alternate strands. Unidirectional deletions in the cloned fragments, of predictable sizes, were produced by use of the Exo/Mung Bean Nuclease Deletion Kit (Stratagene). The DNA sequence was determined by the dideoxynucleotide chain-termination method of Maniatis *et al.* (1982) with the universal M13 forward primer, reverse primer and modified phage T7 DNA polymerase (Sequenase Version 2.0; United States Biochemical Corporation, Ohio, USA). Sequences were analyzed with DNASIS (DNA Sequence Input and Analysis System; Hitachi Software Engineering Co., Ltd., Japan).

RESULTS

Restriction map of the mF plasmid

The purified mF plasmid from the microplasmodia of CH934 x NG7 was digested with appropriate restriction endonucleases and digested fragments were directly cloned into the respective restriction sites in the pBluescript II vector. The restriction map and cloned fragments that were used for the mapping are shown in Fig. 4. The mF plasmid was a linear molecule. By summing the sizes of the restriction fragments, the size of the mF plasmid was estimated to be greater than 11.6 kbp at least. The precise size could not be determined, because the mF plasmid had ends of variable length (see below).

Structure of the ends of the mF plasmid

To determine the structure of the ends and to confirm the linearity of the mF plasmid, the total DNA from the isolated mitochondria was

digested for increasing lengths of time with exonuclease *Bal*31 which attacks double-stranded DNA. This enzyme progressively shortens DNA molecules from their ends and, hence, sequences that are at the ends of the mF plasmid will be progressively shortened, while internal DNA sequences will be unaffected by moderate digestion. After the treatment with *Bal*31, the samples were digested with the restriction endonuclease *Xba*I. The digests were fractionated by electrophoresis and blotted on a nylon membrane. To identify the *Xba*I restriction fragments that originated from each end, the regions near each end in the restriction map (a and b in Fig. 4) were allowed to hybridize to the nylon membrane.

In the case of the restriction pattern of the mF plasmid that was not treated with *Bal*31, hybridization patterns generated by both probes showed banding (Fig. 5). Other restriction endonucleases used instead of *Xba*I gave similar hybridization patterns (Fig. 6). These results suggested that the plasmid contained repeated sequences with a repeating unit at both ends. The number of bands indicated that the most extensive region of repeats included at least 17 repetitions of the repeating unit. Moreover, Southern hybridization patterns of *Bal*31/*Xba*I restriction fragments revealed the degradation of these repeats (Fig. 5). These results showed that the repeated sequences were located at or very near the ends of the mF plasmid, and they also confirmed the linearity of the mF plasmid.

I have tried an experiment with 5' specific λ exonuclease and 3' specific exonuclease III. Both ends of the mF plasmid were sensitive to exonuclease III but insensitive to λ exonuclease (Fig. 7). It seems that the 5' ends of the mF plasmid were protected by terminal structures such as terminal proteins.

Size of the mF plasmid

Considering the extent of these repetitions, the size of the mF plasmid was determined as follows. Since the mF plasmid had two *Xba*I restriction sites, the plasmid consisted of a 9.6-kbp internal fragment and two end-fragments of heterogeneous size. The shortest left end was 2.1 kbp in length and the longest one was more than 4.6 kbp in length. The shortest right end was 1.6 kbp in length and the longest one was more than 4.1 kbp in length. Thus, the size of the mF plasmid was estimated to vary from 13.3 kbp to more than 18.3 kbp.

Terminal inverted repeats of the mF plasmid

The mF plasmid had the unique structure at both ends. To clone the restriction fragments derived from the ends of the mF plasmid, the total mtDNA, including the mF plasmid, was treated with T4 DNA polymerase to blunt the ends of linear DNA molecules, and then digested with the restriction endonuclease *Xba*I. The sample was subjected to electrophoresis and restriction fragments of approximately 2.0 kbp to 2.5 kbp were purified from the agarose gel, since the size of *Xba*I restriction fragments derived from the terminal region of the mF plasmid is estimated to vary from 2 kbp to over 8 kbp (Fig. 5). These fragments were cloned into the *Xba*I/*Sma*I site of the pBluescript vector. Two clones, NPT4X-34 and -64, were chosen for determination of the DNA sequences of the left and right ends of the mF plasmid on the map in Fig. 4.

The sequences of the clones NPT4X-34 and -64 revealed that the mF plasmid had three TIRs at both ends (Fig. 8). The innermost TIR was 591 bp long (926-1516 on the left end; 1214-1804 on the right end in Fig. 8). This 591-bp TIR started from 5' AATGTGAAAT 3' and stopped at 5' CACTACTTCT 3'. Outside of the 591-bp TIRs were 144-bp repeating

units (the boxed sequences in Fig. 8). The clones NPT4X-34 and -64 included 5 and 7 of these 144-bp repeating units, respectively. The sequences of these repeating units were completely identical and were arranged as a kind of TIR (Fig. 8). The 144-bp repeating units started from 5' TAATATAAAT 3' and stopped at 5' CCTTATTGAA 3'. To detect the minimum number of such repeats, I determined the sequences of the terminal fragments from some different clones. Although I found a terminal fragment that had only one 144-bp repeating unit, I could not find a terminal fragment that had no such units.

Outside of this repeat, a 205-bp TIR was located at the extreme ends of the mF plasmid (1 to 205 in both ends in Fig. 8). This TIR started from 5' GTGTTACATT 3' and stopped at TTGTATTGAA 3'. The sequence of the last 7 nucleotides of this 205-bp TIR, 5' TATTGAA 3', was identical to that of the 144-bp repeating unit. Whereas the GC content of the 205-bp TIR was 49%, those of the other TIRs of the mF plasmid were about 23%. Three large hairpin structures were found in this region (Fig. 9). The lengths of the stems of these hairpin structures were 20, 16 and 17 bp, and the theoretical free energies were -29.7, -31.2 and -53.5 kcal/mol, respectively. The total size of these three TIRs is 794 + 144n bp, where n is the number of 144-bp repeating units (from 1 to over 17).

Since T4 DNA polymerase has a 3' to 5' exonuclease activity that can remove protruding nucleotides from the 3' termini of linear DNA, a few nucleotides may have been removed from the ends of the mF plasmid by the treatment with T4 DNA polymerase. To detect the presence of nucleotides on the plasmid's ends, an oligonucleotide primer, 5' GGGGATAATCTATGATC 3' (from 138 to 122 in the nucleotide sequence of the ends in Fig. 8), was used for direct sequencing of the ends

of the mF plasmid. However, since the palindrome structures of the 205-bp TIR may have prevented this direct sequencing, I can not exclude the possibility that nucleotides exist at the extreme ends.

An ORF which overlaps two TIRs may encode DNA polymerase

A search for ORFs was performed by translating the sequences near and within the TIRs in all six possible frames with a universal code. This search yielded one coding sequence of significant length, ORF-547, in the right end of the mF plasmid on the map (Fig. 10). This ORF runs through the 591-bp TIR and terminates in the 144-bp repeating unit. ORF-547 begins at four ATG codons. If the first ATG is assumed to be the translation initiation codon, then ORF-547 encodes a polypeptide of 547 amino acids, including four methionines at the N-terminus. When this amino-acid sequence was compared to the amino-acid sequences in the EMBL and NBRF databases, it showed extensive homology with the putative DNA polymerases that were encoded on other linear mitochondrial plasmids (Fig. 11; see DISCUSSION).

DISCUSSION

Terminal structure of the mF plasmid

The problem of completing the replication of a linear DNA molecule has been recognized for a number of years. All known DNA polymerases synthesize DNA in the 5' to 3' direction and all require a primer that is usually removed; multiple rounds of DNA replication would result in the progressive loss of DNA sequence from the ends. To resolve this general problem, all known linear mitochondrial plasmids have TIRs with terminal proteins covalently linked to the 5' ends of the plasmid (for reviews, see Samac and Sally 1989 and Meinhardt *et al.* 1986). By

contrast, the terminal structure of the mitochondrial plasmid from *P. polycephalum* is different from that of other linear plasmids.

The mF plasmid had two TIRs of 591 and 205 bp long, respectively, which were on either side of one or more 144 bp repeats. The number of 144-bp repeating units varied from one to over 17, and this variation resulted in mF plasmids of different lengths. Repeated arrays at the extreme ends of linear chromosomes are known as telomeres (for reviews, see Blackburn and Szostak 1984; Zakian 1989). In mitochondria, long repeating units, which are estimated to range in length from 31 bp to 53 bp, have been reported in *Tetrahymena* (Morin and Cech 1986, 1988). However, the 144-bp repeating units of the mF plasmid are not located at the extreme ends. In this respect, they are quite different from telomeres. The resistance of the plasmid's ends to 5' specific λ exonuclease (Fig. 7) suggests that the 5' end may be protected by terminal proteins similar to those found in other linear mitochondrial plasmids. The structure of the extreme ends (TIR with a terminal protein) suggests that the mF plasmid utilizes a mode of replication similar to those of *Bacillus* phage φ29 and adenoviruses. Although the structures of the extreme ends are similar to those found in other linear mitochondrial plasmids, repeats similar to the 144-bp repeating units have not been reported in other linear mitochondrial plasmids, linear phages or viruses.

The mechanism that causes the repeating units to occur in varying numbers is still unexplained. The sequence of the last 7 nucleotides of the 205-bp TIR, 5' TATTGAA 3', was identical to that of the 144-bp repeating unit. The heterogeneity may be the result of the 'jumping' of the DNA polymerase from the 5' TATTGAA 3' sequence of the 144-bp repeating units to the identical sequence of the 591-bp TIR. If the replication system of the mF plasmid is similar to that of φ29 and adenoviruses, then replication starts at the extreme ends and progresses

inward. The DNA polymerase first replicates the 205-bp TIR and then proceeds to the 144-bp repeating units. If the DNA polymerase on the 5' TATTGAA 3' sequence of the 144-bp repeating unit jumps to the identical sequence of the 205-bp TIR, it re-replicates the 144-bp repeating units, which means that the replicated mF plasmid will have more of the repeating units than the original plasmid. Moreover, inter- and intra-recombination between this 7-bp nucleotide sequence may also produce different numbers of repeating units.

The sequences of the three TIRs, including those of the 144-bp repeating units, have no significant sequence homology with TIRs of other linear mitochondrial plasmids. The 205-bp TIR located at the extreme ends had a higher GC content than the other TIRs and another sequenced region of the mF plasmid, and could also form three thermodynamically stable hairpin structures. The mF plasmids may require protection against digestion from the ends by exonucleases, and/or recognition of DNA polymerase and a possible terminal protein by its replication system.

ORF-547 encoding DNA polymerase

ORF-547 showed extensive homology with the putative DNA polymerases that were encoded on other linear mitochondrial plasmids (Fig. 11). ORFs encoding DNA polymerases have been described in other linear mitochondrial plasmids, such as S1 in maize (Kuzumin and Levchenko 1987), pCIK1 in *Claviceps purpurea* (Oeser and Tudzynski 1989) , pAI2 in *Ascobolus immersus* (Kempken *et al.* 1989), pMC3-2 in *Morchella conica* (Rohe *et al.* 1991), *kalilo* in *Neurospora intermedia* (Chan *et al.* 1991), pEM of *Agaricus bitorquis* (Robison *et al.* 1991), *maranhar* in *N. crassa* (Court and Bertrand 1992), and pAL2-1 in *Podospora anserina* (Hermanns and Osiewacz 1992). Two conserved

domains are characteristic of the proof-reading and polymerization motifs of DNA polymerases of linear plasmids and linear phages (Bernad *et al.* 1989, Oeser and Tudzynski 1989, Court and Bertrand 1992). The proof-reading domain, which has 3' to 5' exonuclease activity, is located at the N-terminus and is characterized by three amino-acid sequence blocks, Exo I, Exo II and Exo III (Bernad *et al.* 1989). Three strongly conserved blocks (Pol I, Pol II and Pol III) are located in the C-terminal polymerization domain (Oeser and Tudzynski 1989, Court and Bertrand 1992). Figure 11 shows that ORF-547 contained these three polymerization blocks but did not contain the three conserved blocks of the proof-reading domain. These results suggest that the DNA polymerase encoded by ORF-547 had the DNA polymerizing activity, but not the proof-reading activity. The known DNA polymerases that are encoded in other linear mitochondrial plasmids all contain 3' to 5' exonuclease domains (Court and Bertrand 1992, Hermanns and Osiewacz 1992). I tried to use the mitochondrial genetic code of filamentous fungi (TGA coding for Trp; Fox 1987), but could not find ORFs larger than ORF-547. Moreover, amino acid sequences derived from the other possible frames, including stop codons, were compared with the 3' to 5' exonuclease domain, but none of these frames encoded the exonuclease domain. Therefore, there are two hypotheses with regard to the proof-reading activity of the DNA polymerase of the mF plasmid. One hypothesis is that the DNA polymerase encoded by ORF-547 does not have 3' to 5' exonuclease activity, or that it does have exonuclease activity, but without the exonuclease domain. The other hypothesis is that extensive editing of mRNA, such as that in the gene of the α-subunit of ATP synthetases from mitochondria of *P. polycephalum* (Mahendran *et al.* 1991) creates the 3' to 5' exonuclease domain of the DNA polymerase. Further work is needed to analyze the proof-reading activity

of the DNA polymerase encoded by ORF-547.

The left end of the mF plasmid did not contain any long ORFs (>300 amino acids). The longest ORF was encoded in the 591-bp TIR and the 144-bp repeating unit. This ORF started in the 591-bp TIR marked in Fig. 10, if the first ATG codon after stop codons of the same frame can be considered the initiation codon, and encoded a polypeptide of 221 amino acids (ORF-221). Since ORF-221 contained two conserved blocks of the polymerization domain (Pol II and Pol III) but did not include Pol I, it may not function as DNA polymerase.

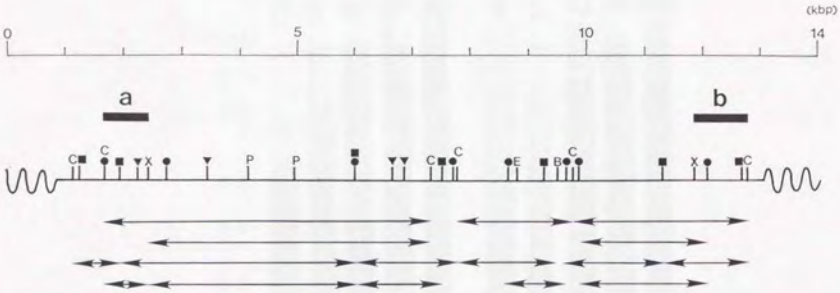

Fig. 4
Restriction map of the mF plasmid from *P. polycephalum*. The symbols X (*Xba*I), ● (*Hin*dIII), ▼ (*Hin*cII), ■ (*Eco*RV), B (*Bam*HI), C (*Cla*I), E (*Eco*RI) and P (*Pst*I) represent the various restriction sites. Terminal regions of size heterogeneity are represented by wavy lines. The regions used as probes in Figs. 5 and 6 are indicated by closed boxes (a and b). The lower part of the Figure shows the location and extent of the fragments cloned in pBluescript.

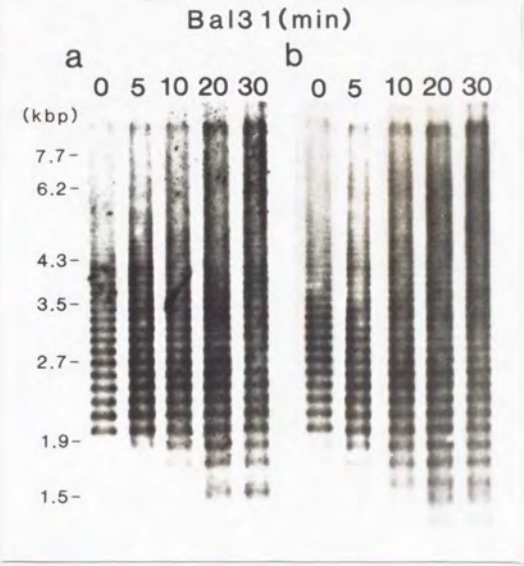

Fig. 5
The sensitivity to exonuclease *Bal*31 of terminal regions of the mF plasmid. The total DNA from mitochondria was digested with *Bal*31 for 0, 5, 10, 20 and 30 min, as indicated. The samples were then digested with *Xba*I and digests were fractionated by agarose-gel electrophoresis. The gel was blotted on a nylon membrane and hybridized with the probes (a and b) indicated in Fig. 4.

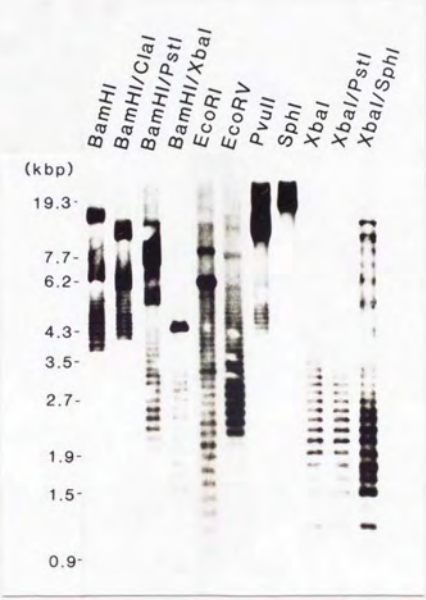

Fig. 6
The total DNA from mitochondria was digested with the restriction enzyme as indicated above each lane. The samples were fractionated by electrophoresis and the gel was blotted on a nylon membrane and hybridized with the probe (a) in Fig. 4.

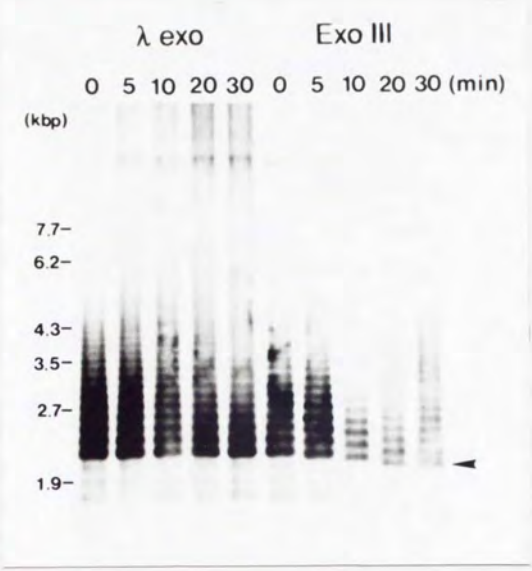

Fig. 7
The sensitivity to λ exonuclease and Exonuclease III of terminal regions of the mF plasmid. The total DNA from mitochondria was digested with each enzyme for 0, 5, 10, 20 and 30 min, as indicated. The samples were then digested with *Xba*I and digests were fractionated by agarose-gel electrophoresis. The gel was blotted on a nylon membrane and hybridized with the probes in Fig. 4.

```
Left  GTGTTACATT TGTAACACCA ATGGTCTACA TATTACCATT GGTGTTATAA ATGTGCTTCT AGCTGTTATA CCCATGGGGT CCGGACCGTG GGTATAACAG  100
Right GTGTTACATT TGTAACACCA ATGGTCTACA TATTACCATT GGTGTTATAA ATGTGCTTCT AGCTGTTATA CCCATGGGGT CCGGACCGTG GGTATAACAG  100

      CGTACATGGG AGGGGGTGGG GGATCATAGA TTATCCCCCA CCCCCTCCCT CCTCCCTCCT CCCTCCTCCC TCTCTCCTCC CTCTTCTTTG TTATATTGTA  200
      CGTACATGGG AGGGGGTGGG GGATCATAGA TTATCCCCCA CCCCCTCCCT CCTCCCTCCT CCCTCCTCCC TCTCTCCTCC CTCTTCTTTG TTATATTGTA  200

      TTGAATAATA TAAATTATTA TTATTATTAT TGAAAAAGGC ATTTTATCTT ATACATATGA TCCATGGAGT AGTTTTATAG TTTGGAATTA ATTTCCTTTT  300
      TTGAATAATA TAAATTATTA TTATTATTAT TGAAAAAGGC ATTTTATCTT ATACATATGA TCCATGGAGT AGTTTTATAG TTTGGAATTA ATTTCCTTTT  300

      TCTTGATGCT TGAAAAGAAT ATGTTTGATT ATTATTCAGC CTTATTGAAT AATATAAATT ATTATTATTA TTATTGAAAA AGGCATTTTA TCTTATACAT  400
      TCTTGATGCT TGAAAAGAAT ATGTTTGATT ATTATTCAGC CTTATTGAAT AATATAAATT ATTATTATTA TTATTGAAAA AGGCATTTTA TCTTATACAT  400

      ATGATCCATG GAGTAGTTTT ATAGTTTGGA ATTAATTTCC TTTTTCTTGA TGCTTGAAAA GAATATGTTT GATTATTATT CAGCCTTATT GAATAATATA  500
      ATGATCCATG GAGTAGTTTT ATAGTTTGGA ATTAATTTCC TTTTTCTTGA TGCTTGAAAA GAATATGTTT GATTATTATT CAGCCTTATT GAATAATATA  500

      AATTATTATT ATTATTATTG AAAAAGGCAT TTTATCTTAT ACATATGATC CATGGAGTAG TTTTATAGTT TGGAATTAAT TTCCTTTTTC TTGATGCTTG  800
      AATTATTATT ATTATTATTG AAAAAGGCAT TTTATCTTAT ACATATGATC CATGGAGTAG TTTTATAGTT TGGAATTAAT TTCCTTTTTC TTGATGCTTG  800

      AAAAGAATAT GTTTGATTAT TATTCAGCCT TATTGAATAA TATAAATTAT TATTATTATT ATTGAAAAAG GCATTTTATC TTATACATAT GATCCATGGA  700
      AAAAGAATAT GTTTGATTAT TATTCAGCCT TATTGAATAA TATAAATTAT TATTATTATT ATTGAAAAAG GCATTTTATC TTATACATAT GATCCATGGA  700

      GTAGTTTTAT AGTTTGGAAT TAATTTCCTT TTTCTTGATG CTTGAAAAGA ATATGTTTGA TTATTATTCA GCCTTATTGA ATAATATAAA TTATTATTAT  800
      GTAGTTTTAT AGTTTGGAAT TAATTTCCTT TTTCTTGATG CTTGAAAAGA ATATGTTTGA TTATTATTCA GCCTTATTGA ATAATATAAA TTATTATTAT  800

      TATTATTGAA AAAGGCATTT TATCTTATAC ATATGATCCA TGGAGTAGTT TTATAGTTTG GAATTAATTT CCTTTTTCTT GATGCTTGAA AAGAATATGT  900
      TATTATTGAA AAAGGCATTT TATCTTATAC ATATGATCCA TGGAGTAGTT TTATAGTTTG GAATTAATTT CCTTTTTCTT GATGCTTGAA AAGAATATGT  900

      TTGATTATTA TTCAGCCTTA TTGAA---- ---------- ---------- ---------- ---------- ---------- ---------- ----------  925
      TTGATTATTA TTCAGCCTTA TTGAATAATA TAAATTATTA TTATTATTAT TGAAAAAGGC ATTTTATCTT ATACATATGA TCCATGGAGT AGTTTTATAG 1000

      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
      TTTGGAATTA ATTTCCTTTT TCTTGATGCT TGAAAAGAAT ATGTTTGATT ATTATTCAGC CTTATTGAAT AATATAAATT ATTATTATTA TTATTGAAAA 1100

      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
      AGGCATTTTA TCTTATACAT ATGATCCATG GAGTAGTTTT ATAGTTTGGA ATTAATTTCC TTTTTCTTGA TGCTTGAAAA GAATATGTTT GATTATTATT 1200

      ---------- ---AATGTGA AATAATGATG ACCTAATGTT ATATTTAATA TTTTCTTGTG TTGTGTAATA ATATCATGTA TGTTGAATAT TGGTTTTTGT 1012
      CAGCCTTATT GAAAATGTGA AATAATGATG ACCTAATGTT ATATTTAATA TTTTCTTGTG TTGTGTAATA ATATCATGTA TGTTGAATAT TGGTTTTTGT 1300

      AATGGTATTC CTTTAAATTT ATAAATAATA GGAGAATTAA TAGGTGCATA AATGTAAAAT TTATTGGCTA TAAAATGAGC TTCTGCGTTG ATAGATTCAA 1112
      AATGGTATTC CTTTAAATTT ATAAATAATA GGAGAATTAA TAGGTGCATA AATGTAAAAT TTATTGGCTA TAAAATGAGC TTCTGCGTTG ATAGATTCAA 1400

      GACGAAATTT GCCCATTTCT TTAGAAGTTG TTAAAGCAAT ATCCGGTATA GGATTCTTTA AAAATAAACC ATCTGTATCT ATATATATAA CATGAAGGTT 1212
      GACGAAATTT GCCCATTTCT TTAGAAGTTG TTAAAGCAAT ATCCGGTATA GGATTCTTTA AAAATAAACC ATCTGTATCT ATATATATAA CATGAAGGTT 1500

      ATAATTTAAA ATAGTGTTAT ACATAAAAAT ACGAGCATAA GATGTTATAG CACTAGTAAT AGCAATATTA TTATAACAAG TATTTGCTGT TATATCGATA 1312
      ATAATTTAAA ATAGTGTTAT ACATAAAAAT ACGAGCATAA GATGTTATAG CACTAGTAAT AGCAATATTA TTATAACAAG TATTTGCTGT TATATCGATA 1600

      AATTCTGTAG TATCATGGGA TATATATGTA TTATCTGTGA TTAATTCTTT TTCAGGTGAT ATGATATCTA TTTGTTCATA GACTAAACCA AAACGACCAT 1412
      AATTCTGTAG TATCATGGGA TATATATGTA TTATCTGTGA TTAATTCTTT TTCAGGTGAT ATGATATCTA TTTGTTCATA GACTAAACCA AAACGACCAT 1700

      ATAATGTATT TAATAATTTT TTATATAAAT GCTTTAATGC AGGATCTTTT GCTTTCAATC TTCTATTATA CATTTGTTCT ACATATTCTT CAAACACTAC 1512
      ATAATGTATT TAATAATTTT TTATATAAAT GCTTTAATGC AGGATCTTTT GCTTTCAATC TTCTATTATA CATTTGTTCT ACATATTCTT CAAACACTAC 1800

      TTCTCTGCTG TATATAGAAT ATTATTATTC AGTACAAATT TTAAACAATA AAAACAAAAA AAATATGAAC AATACCAAAA TAACAAATAT AAAATTAATT 1612
      TTCTTTTTCT TTATATTCAT ATGCGGAATA TATTTCTATT ATTTTATATC CTTGTTTAAG TGCTAATTTT ATTTCTTCTG AAAAATAAAT TCCTCTTAAT 1900
```

205bp 144bp 591bp

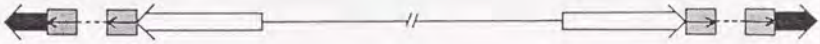

Fig. 8

A comparison of nucleotide sequences of the left and right ends of the mF plasmid. The matches between the left and right ends are indicated by asterisks. There is 205-bp TIR at extreme ends, which is shown as a closed arrow in the lower illustration. The 144-bp repeating unit is boxed and is represented as the shaded boxes in the lower illustration. The clones NPT4X-34 (derived from the left end) and -64 (derived from the right end) had 5 and 7 repeating units, respectively. The sequences of each repeating unit were perfectly identical. Inside these repeats, a 591-bp TIR is shown as an open arrow in the lower illustration.

```
                                                  A T G
                                                T       T
                                                A       A
                                                 A     G
                    C T A                          T-A
                  T       G G A                    G-C
                A       T C                        G-C
                 A     G C       C                 T-A
                  T-A       T     C                A-T
                  A-T       G-C                    A-T
                  G-C       G-C                    C-G
                  G-C      C   A                   C-G
                  G-C       A-T                    A-T
                  G-C       C-G                    C-G
                  G-C       C-G                    A-T
                  T-A       C-G                    A-T
                  G-C       A-T                    T-A
                  G-C       T-A                   A   C
                  G-C       A-T                    T-A
                  G-C       T-A                    T-A
                  A-T       G-C                    T-A
                  G-C       T-A                    A-T
                  G-C       C-G                    C-G
                  G-C       G-C                    A-T
—TTCAATACAATATAACAAAGAAGAGGGAGGAGAGAGGGAGGAGGGAGGAGGGAGGA   ATGTAC   TAGAAGC   AACAC-3'
```

Fig. 9
The putative secondary structure of the 205-bp TIR. Thermodynamically-favored secondary structures were determined for the 205-bp TIR.

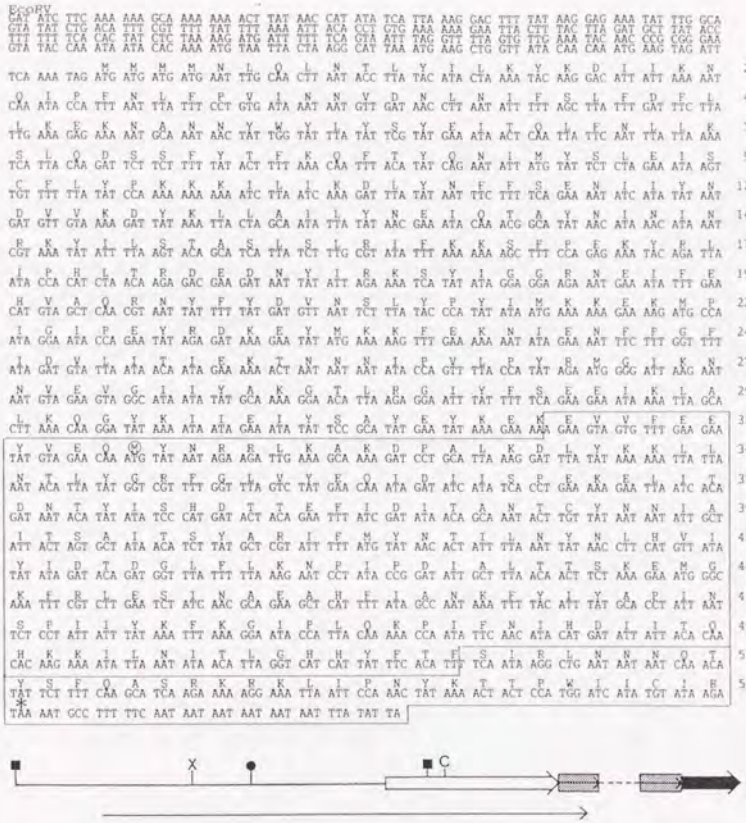

Fig. 10
Amino-acid sequence of putative DNA polymerase coding in the right end on the map of the mF plasmid. The nucleotide sequence between the *Eco*RV site (■in the lower enlarged map of the right end) and the end of only one 144-bp repeating unit is shown. The nucleotide sequences of the 591-bp TIR and the 144-bp repeating unit are boxed. The stop codon is represented by an asterisk. The characters and symbols in the lower drawing are the same as those in Figs. 4 and 8. The region and direction of ORF-547 are indicated by the arrow below the schematic map.

```
                Pol I                                  Pol II                                 Pol III
pMC3-2    526   CIKT-KSYDCNSIYPYCMLKDMPVEN   652   PTAKLLLNGLYGRFGMNP   763   KVFMTDTDCIWMNGSLS
                 *  . ** **.**.     **  .                . .* ***.* *****                .. **** *  .  .
pAL2-1    667   YGKNLRYYDVNSTYPFVAKNTMPGHE   793   TMTKFLLNSLLGRFGMSM   913   NLYYTDTDSIVTDIDTP
                ..*   ****** ***.. * .**                ..*  .****.*****. .                 . **********.  .*
pC1K1     697   STKSYYYYDVNSLYPFASINDIPGLK   827   NIAKLILNSLIGRFGMNI   946   TLYYTDTDSIVTDLKLF
                 ..    ********* . ..*.*  *                 * *. .*** ****.                  .*******.* .  **
S-1       487   YGENLYYYDVNSLYPSSMLDDMPIGK   614   FIYKITMNSLYGRFGISP   712   DCYYTDTDSVVVERELP
                 ..    *.********  . ..                 .** .*.******.                   * ***.. .  .*
mF        198   VAQRNYFYDVNSLYPYIMKKEKMPIG   341   DLYKKLLNTLYGRFGLVY   420   HVIYIDTDGLFLKNPIP
                 .      **.*****  * *  ** *                 . * *.*.******.                 . .****.  .  *
pAI2      814   EGKNIHSYDINSLYPSAMAKFDMPTG   947   FIAKLLMNSLYGRFGMDP   039   NLYAVDTDGIKVDTEID
                  * **.***.***** **   * **.*                 .*.*********.. .*                 *.*  .*******. ..*
kalilo    627   FGVNIKSYDVNSLYPFAMKYFKMPSG   768   YISKLLMNSLYGRFGLNP   860   NIYYIDTDGIKVDIDLD
                     ** *.* ***** **     ** *                 ..***.*.**** *.*                 *  * ***.* *.  **
maranhar  620   IINNIFSFDFNSLYPTAMM-MPMPVG   748   QMAKLLLNTLYGRTGMND   857   NSAYTDTDSIFVEKPLD
                 .. *  . .* ** ** **.    ** *                 .*** **.***. *  .                 .  . ..***. . ***.
pEM       436   LVKNGYHYDMNSQYPYAML-QSMPTG   569   YIAKLSLNSLYGKFGQKE   688   LAIASNTDSLILRKPLE
```

Fig. 11
ORF-547 protein contains the polymerizing domains of DNA polymerases. The DNA polymerases shown here are as follows: the linear mitochondrial plasmids, pMC3-2 (Rohe *et al.* 1991), pAL2-1 (Hermanns and Osiewacz 1992), pCIK1 (Oeser and Tudzynski1989), S1 (Kuzumin, and Levchenko 1987), pAI2 (Kempken *et al.* 1989), *kalilo* (Chan *et al.* 1991), *maranhar* (Court and Bertrand 1992) and pEM (Robison *et al.* 1991). The sequences have been aligned for maximum similarity. Identical amino acids and conserved exchanges between amino acids in neighboring sequences are indicated by asterisks and dots, respectively. The numbers of the first amino acid of each block are indicated to the left of each of the amino-acid sequences.

CHAPTER II

Constitutive homologous recombination between mitochondrial DNA and a linear mitochondrial plasmid in *P. polycephalum*

SUMMARY

In one particular myxamoebal strain (NG7; mF^+) of *P. polycephalum*, a linear mitochondrial plasmid (mF) which promotes mitochondrial fusion has been identified. While a mating between mF^- strains that do not carry the mF plasmid resulted in uniparental inheritance of the mtDNA, in matings between mF^+ and mF^- strains a recombination occurred between the mtDNA and the mF plasmid, and recombinant mtDNA with the ends of the mF plasmid as its ends was generated. The DNA sequences of the recombination site in the mtDNA and the mF plasmid and of the recombinant mtDNA revealed that the mF plasmid had a 475-bp sequence that was identical to, but slightly shorter than, a 479-bp sequence of the mtDNA. This so-called identical sequence was found at the junction between unique sequences of the mF plasmid and the mtDNA in the recombinant mtDNA. Thus, the recombination between the mtDNA and the mF plasmid was due to reciprocal crossing-over at the identical sequence.

INTRODUCTION

In *P. polycephalum* the extent of variation in mtDNA among strains is very high, as compared to that found in other organisms (Kawano *et al.*

1987). Variations in mtDNAs among strains have also been observed in many fungi, and the extent of such variation is generally greater than that in animal mtDNAs. Polymorphism of animal mtDNAs results predominantly from single-base substitutions, but it has been postulated that the variations in fungi are the results of insertions or deletions from basically similar genomes (Turner *et al.* 1982). However, the variation among mtDNAs of *P. polycephalum* seems to be significantly greater than that in other fungi.

Generally, structural divergence of mitochondrial genomes is due to the following features of these genomes: (1) amplification of a specific sequence in the mtDNA (*e.g.*, *petite* mutants of yeast and plasmid-like DNAs of senescent strains of *Podospora*); (2) insertions of introns, (*e.g.*, intron ω in *Saccharomyces cerevisiae*); (3) inversion of sequences by recombination between inverted repeats; and (4) formation of subgenomic molecules by recombination between tandem repeats in circular mtDNA (for reviews, see Sederoff 1984; Wolf and Del Giudice 1988; Lonsdale 1989). Moreover, in many species, mitochondria carry, in addition to a high-molecular-weight DNA component that represents the main mtDNA, smaller circular and linear DNA molecules as mitochondrial plasmids (for reviews, see Samac and Leong 1989; Lonsdale 1989; Meinhardt *et al.* 1990). These plasmids replicate autonomously in the mitochondria independently of the mtDNA and may be responsible for the diversity among mitochondrial genomes. It has been demonstrated that some of these plasmids are integrated into mtDNA and cause structural changes in the mtDNA (Myers *et al.* 1989; Lonsdale 1989; Court *et al.* 1991).

A mitochondrial fusion-promoting plasmid, named mF, was first identified in the myxamoebal strain NG7 (Kawano 1991a; Kawano *et al.*

1991a). In CHAPTER I, I demonstrated that the mF plasmid had three TIRs including repeating units at both ends and its size was estimated to vary from 13.3 kbp to more than 18.2 kbp, depending on the extent of repetitions. The mtDNA from CH934 (mF^-) is M (McArdle) type and its restriction map was reported previously (Takano *et al.* 1990). The mtDNA of the M type is a linear molecule of 86.0 kbp with a tandem duplication of 20.0 kbp near each end. In matings between the mF^- myxamoebal strains that do not carry the mF plasmid, transmission of the mtDNA to plasmodia is uniparental; plasmodia of *P. polycephalum* always carry mtDNA with the restriction pattern of only one of the two parental types (Kawano *et al.* 1987; Kawano and Kuroiwa 1989). In matings between the mF^+ strain that carries the mF plasmid and the mF^- strain, however, transmission of the mtDNA does not occur via simple uniparental inheritance: the plasmodia formed in such matings give novel restriction fragments, as well as the restriction fragments that are characteristic of the parental mtDNAs and the mF plasmid.

In this CHAPTER, I present evidence that the structural changes in the mtDNA during the formation of the plasmodia were due to constitutive homologous recombination between the mtDNA and the mF plasmid.

MATERIALS AND METHODS

Strains and culture conditions were obtained as described in MATERIALS AND METHODS of CHAPTER I.

The mtDNA and the mF plasmid were isolated by the methods of Kawano *et al.* (1991a). The digestion with restriction endonuclease, cloning techniques and construction of the restriction map were performed as described previously (Takano *et al.* 1990).

For the DNA sequence of the homologous region of the mtDNA, the mF plasmid and the recombinant mtDNA, the *Hin*cII/*Cla*I fragment from 2.4 kbp in the mtDNA, the *Bam*HI/*Eco*RV fragment from 1.8 kbp in the mF plasmid, and the *Hin*cII/*Eco*RV fragment of 2.6 kbp and the *Hin*dIII/*Cla*I fragment of 1.3 kbp from the recombinant mtDNA (Fig. 13) were subcloned from existing clones. Sequence was carried out according to the methods in CHAPTER I.

The treatment with *Bal*31, agarose gel electrophoresis, blotting on a nylon membrane and Southern hybridization with the ECL Gene-Detection System (Amersham International plc) were performed as described previously (Takano *et al.* 1990). Probes for Southern hybridization were prepared from the cloned fragment of the mtDNA and the mF plasmid as described previously (Takano *et al.* 1990).

RESULTS

Transmission of the mtDNA to plasmodia

I reexamined the pattern of transmission of mtDNA during formation of plasmodia by mating pairs of myxamoebal strains that carried mtDNAs that were distinguishable in terms of RFLP. In the mating between CH934 (mF^-) and NG7 (mF^+), the pattern of transmission of the mtDNA was not straight forward (Fig. 12A) because the mF plasmid was also transmitted to the resultant plasmodia (CH934 x NG7). The *Hin*dIII digests of the mF plasmid contained the 3.2-, 2.3-, 1.7-, 1.0-, 1.0-, 0.8- and 0.3-kbp internal fragments and two terminal fragments of

heterogeneous size. The mF plasmid of NG7 was transmitted to the plasmodia without any changes (*i.e.*, the 3.2-kbp fragment). However, the mtDNA of NG7 was not transmitted to the plasmodia (*i.e.*, the 13.5-kbp fragment of NG7). Moreover, novel fragments of 11.0 kbp and 6.5 kbp appeared in the restriction pattern of the mtDNA from CH934 x NG7, as reported previously by Kawano *et al* (1991a). These changes of the restriction pattern suggest that recombination occurred between the two species of mtDNA and/or the mF plasmid during the formation of plasmodia.

Recombination between the mF plasmid and mtDNA

To analyze the pattern of transmission of the mtDNA, the novel restriction fragments in CH934 x NG7 (the 11.0- and 6.3-kbp *Hin*dIII fragments; Fig. 12A), which were not found in the restriction pattern of the mtDNA of CH934, were cloned into the pBluescript vector. The restriction sites on the novel restriction fragments were compared with those of the mtDNA of the M type and those of the mF plasmid for which a restriction map has also been constructed (CHAPTER I). These fragments consisted of one part that was identical to the duplication of the mtDNA of the M type and one part that was identical to the mF plasmid (Fig. 13). These results suggest that the novel fragments were generated by recombination between the duplication of the mtDNA of the M type and the mF plasmid. The recombination occurred between the region about 11.0 kbp from the left end of the duplication and the region about 3.0 kbp from the right end of the mF plasmid on the maps. The recombination may be caused by reciprocal crossing-over between the mtDNA of the M type and the mF plasmid.

To construct the restriction map of the recombinant mtDNA from

CH934 x NG7, all *Xba*I restriction fragments, with the exception of the 0.9- and 0.3-kbp fragments and the terminal fragments, were cloned into the pBluescript vector. All cloned *Xba*I restriction fragments and the largest *Hin*dIII fragment (15.5 kbp) were identical with the corresponding restriction fragments of the M type. It appeared that these fragments were transmitted from CH934 (Fig. 12A). By contrast, no unique restriction fragments from the mtDNA of NG7 were included in the restriction pattern of the recombinant mtDNA from CH934 x NG7 (*i.e.*, the largest *Hin*dIII restriction fragment of 13.5 kbp; Fig. 12A). These results excluded the possibility that recombination had occurred between the two mtDNA species from the parents, CH934 and NG7, because most of the recombinant mtDNA in CH934 x NG7 was transmitted only from CH934.

To confirm that recombination occurred between the duplication of the mtDNA and the mF plasmid, the restriction fragments near the site of recombination in the duplication of the mtDNA of CH934 (a and b in Fig. 13) were hybridized to the fragments from *Hin*dIII digestion of the mtDNAs of CH934, NG7 and CH934 x NG7 (Fig. 12B and 12C). In the case of the restriction pattern of the mtDNA from CH934, each probe hybridized only to the band of 15.5 kbp derived from the duplication of the mtDNA of CH934 (lane 1 in Fig. 12B and 12C). By contrast, in CH934 x NG7, two bands hybridized with each probe. One band (15.5 kbp) was derived from the duplication of the mtDNA (15.5 kbp) and the other band was derived from the region that crossed over with the mF plasmid (11.0 kbp in Fig. 12B and 6.5 kbp in Fig. 12C). In the case of NG7, each probe always hybridized to two bands: 13.5 kbp and 11.0 kbp, in Fig. 12B; and 13.5 kbp and 4.3 kbp, in Fig. 12C. The hybridization patterns of the mtDNA of NG7 revealed a 2.0-kbp deletion in the duplicated region, as compared with that of CH934. The deletion is

located to the right of the recombination site in the duplication. Thus, in NG7, the band (4.5 kbp) that seemed to be generated by the recombination between the mtDNA of NG7 and the mF plasmid represented a smaller fragment than that from CH934 (6.5 kbp). The fragment containing the recombination site in the mF plasmid (c in Fig. 13) was hybridized to the bands from *Hin*dIII digestion (Fig. 12D). In the case of CH934 x NG7, the probe hybridized to a 2.3-kbp band derived from the mF plasmid, the two novel bands (11.0 kbp and 6.5 kbp) of the recombinant mtDNA and the band (15.5 kbp) derived from the duplication. This 15.5-kbp band, which was present in the restriction pattern of CH934, hybridized weakly with the probe. This observation suggests that the duplication of the mtDNA included a region homologous to the mF plasmid and that the crossing-over between the mtDNA and the mF plasmid occurred at these homologous regions.

Homologous regions in the mF plasmid and mtDNA

To analyze the recombination between the mtDNA and the mF plasmid, the homologous regions of the mtDNA and the mF plasmid, and the recombination site of the recombinant mtDNA were sequenced (Fig. 14). The mF plasmid had a 475-bp sequence that was identical to a 479-bp sequence of the mtDNA with the exception of deletions of a total of four base pairs at three sites. The sequence identical to that of the mtDNA started from nucleotides 5' TAAAAGAAA 3' and stopped at 5' TTTGTTTG 3' in the duplication of the mtDNA. By contrast, the identical sequence in the mF plasmid started at nucleotides 5' TAAAGAAA 3' with a one-base deletion and stopped at 5' TTTGTTTG 3', which is same as the sequence in the mtDNA. Other deletions in the mF plasmid corresponded to positions 22 and 91-92 of the mtDNA in Fig.

14. The recombinant mtDNA (11.0- and 6.3-kbp *Hin*dIII restriction fragments of the recombinant mtDNA) also included the so-called identical sequence (Fig. 13). In the 11.0-kbp *Hin*dIII fragment (R1 in Fig. 14), the left region from the identical sequence was same as that of the mtDNA and the right region was same as that of the mF plasmid. Moreover, its identical sequence was 479 bp long and of the mtDNA type. By contrast, in the 6.5-kbp *Hin*dIII fragment (R2 in Fig. 14), the left region from the identical sequence was same as that of the mF plasmid and the right region was same as that of the mtDNA. Its identical sequence was 475 bp long and of the plasmid type. These results show that recombination occurred at a region between positions 92 and 479 in the identical sequence of the mtDNA type. Such a long common sequence suggests that the homologous recombination between the mtDNA and the mF plasmid is due to crossing-over at these sequences.

Terminal structure of the recombinant mtDNA

To analyze the terminal structure of the recombinant mtDNA, the total DNA from the isolated mitochondria of CH934 x NG7 was digested for increasing lengths of time with exonuclease *Bal*31. After the treatment with *Bal*31, the samples were digested with the restriction endonucleases *Eco*RI and *Pst*I. The digests were fractionated by electrophoresis and blotted on a nylon membrane. To identify the restriction fragment that originated from the right end of the recombinant mtDNA, a 0.6-kbp *Pst*I/*Hin*cII restriction fragment (a in Fig. 13; closed boxes in Fig. 15) was hybridized to the membrane (Fig. 15). The probe hybridized to bands of 4.2 kbp and about 5.3 kbp. The band of 4.2 kbp is derived from the duplication, while the diffuse band of about 5.3 kbp is derived from the recombinant mtDNA that has crossed over with the mF plasmid (Fig.

15). The probe distinguished the duplication that had not crossed over with the mF plasmid from the recombinant mtDNA. The diffuse band was due to extensive repeats of the ends of the mF plasmid that were located at the ends of the recombinant mtDNA (see CHAPTER I). The densitometric scanning of the two bands showed that the fragments were present in approximately equal amounts. Thus one-half of the duplication of the mtDNA was recombined with the mF plasmid. With increasing duration of the treatment with *Bal*31, the diffuse band was affected by *Bal*31, but the band of 4.2 kbp was unaffected (Fig. 15). The sensitivity to *Bal*31 exonuclease of the diffuse band demonstrated that the end of the mF plasmid with extensive repeating units is located at the terminus of the recombinant mtDNA.

DISCUSSION

Transmission of the mF plasmid and the recombinant mtDNA, and mitochondrial fusion

The mF plasmid is apparently responsible for promotion of fusion of mitochondria in zygotes (Kawano *et al.* 1991a). Thus, I can explain the transmission of the mF plasmid and the recombinant mtDNA in the mating between CH934 (mF^-) and NG7 (mF^+) as follows (Fig. 16). The mitochondria of two parental myxamoebal strains fuse with each other in the zygote. The two parental mtDNAs of mF^+ and mF^-, and the mF plasmid can be paired in one mitochondrion, and then crossing-over occurs between the mtDNA of mF^- and the mF plasmid. In every mating between CH934 (*mat*A2) and NG7 (*mat*A12), the recombinant mtDNA from the crossing over between the mtDNA of mF^- and the mF plasmid is transmitted to the plasmodium. When the mF^- strain is changed from

CH934 to OX110 (*mat*A2), the same pattern of transmission of the recombinant mtDNA is obtained, as shown previously (Kawano *et al.* 1991a). It seems that the recombinant mtDNA is transmitted according to the *mat*A hierarchy (*mat*A2 > *mat*A12) with respect to the transmission of the mtDNA (Kawano *et al.* 1987; Meland *et al.* 1991). The data in Fig. 12 suggest that the mtDNA of mF^+ (NG7) crosses over with the mF plasmid. Thus, whichever mtDNA, from mF^- or from mF^+, is transmitted to the plasmodium, the sequence of the mF plasmid is maintained in the next generation as part of the recombinant mtDNA. Moreover, free plasmid that is not crossed over with the mtDNA in the fused mitochondrion is preferentially transmitted to the plasmodium regardless of the transmission of the mtDNA. The fusion of mitochondria is important and provides the basic mechanism for the spreading of the mF plasmid and the recombinant mtDNA with the mF plasmid through the mitochondrial population. The fact that the mF plasmid is associated with mitochondrial fusion gives it an advantage in terms of the frequency with which it is transmitted.

Recombination between the mF plasmid and the mtDNA

The sequences of recombination sites indicated that the mtDNA and the mF plasmid had long identical sequences at which they could cross over reciprocally. Integrations of linear plasmids have been found in the mtDNAs of *Neurospora* (Myers *et al.* 1989; Court *et al.* 1991), maize (Schardl *et al.* 1985), *Claviceps purpurea* (Tudzynski and Esser 1986). Among these plasmids, *kalilo* of *N. intermedia* has been well studied. It induces senescence in *Neurospora* by integrating into the mtDNA (Bertrand *et al.* 1985; 1986). The integration of *kalilo* occurs at 7 distinct regions of the mtDNA and always generates very long inverted

repeats of mtDNA flanking the two ends of *kalilo* insertion sequence (Dasgupta *et al.* 1988). In the case of the mF plasmid, however, the identical sequence between the mtDNA and the mF plasmid was not flanked by inverted repeats of the mtDNA and the recombination between the mtDNA and the mF plasmid was not associated with senescence of *P. polycephalum*.

The comparison between the so-called identical sequences of the mtDNA and the mF plasmid showed a high level of similarity in nucleotide sequence (more than 99%), which suggests a common origin for this sequence. With respect to the origin of the identical sequence in mtDNA and plasmids, the S1 and S2 plasmids have been well studied (for review, see Lonsdale 1989). Southern hybridization and sequence studies suggest that the sequence in the mtDNA that is identical to that in the S1 and S2 plasmids is derived from the R1 and R2 plasmids, which are plasmids that are closely related to the S1 and S2 plasmids (Schardle *et al.* 1984, 1985; Houchins *et al.* 1986). The R1 and S1 plasmids exhibit approximately 70% homology and R2 and S2 exhibit complete sequence homology (Weissinger *et al.* 1982). The R1 and R2 plasmids presumably integrated into the mtDNA and became fixed as sequence identical to the S1 and S2 plasmids after deletion of other sequences, concomitant with the loss of the free plasmids. From such speculation, it seems possible that, in mitochondria of *P. polycephalum*, the mF plasmid may have recombined with the mtDNA in an ancient strain and become fixed as the so-called identical sequence after deletion of the entire sequence apart from the identical sequence and loss of the free plasmid. This hypothesis is supported by the fact that the identical sequence of the mF plasmid hybridized with the mtDNA of all strains during Southern hybridization (see Fig. 23).

Structural conversion of mtDNA

The constitutive reciprocal crossing-over between the mtDNA and the mF plasmid caused structural changes in the mtDNA. The mF plasmid crossed over with the duplication of the mtDNA, so that three types of structural conversion of the mtDNA were possible: crossing-over at the left duplication, at the right duplication and at both duplications (Fig. 17). Each crossing-over creates large and small recombinant mtDNAs. As shown in Fig. 15, one-half of the duplication of the mtDNA was recombined with the mF plasmid, the small recombinant mtDNAs derived from the left or right duplications should be detectable by Southern hybridization. I hybridized left and right sequences, with respect to the recombination site in the duplication of the mtDNA, to DNA after electrophoresis of untreated DNA isolated from the mitochondria of CH934 x NG7. The sequences hybridized to low-molecular-weight fragment as well as to a larger fragment, but the hybridization patterns were smeared and browed. This result was due to the repeating unit at the ends of the mF plasmid that was joined to the recombinant mtDNA and the degradation of the recombinant mtDNA by nucleases of *P. polycephalum* during isolation. From this experiment, I was unable directly to determine the proportion of the recombinant mtDNAs to the mtDNA.

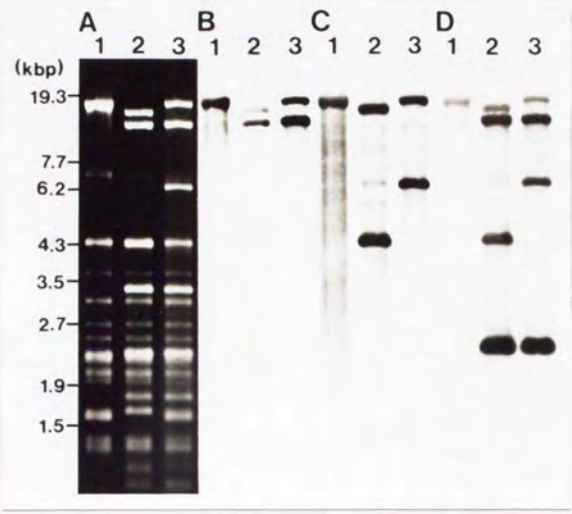

Fig. 12
Pattern of transmission of mtDNA to plasmodia in a mating between CH934 (mF^-) and NG7 (mF^+). Lanes 1, 2, and 3 show the *Hin*dIII restriction patterns of the parental amoebal strains (1 and 2), and the plasmodia (3). Panels show the pattern of staining with ethidium bromide (A); the pattern of hybridization with (B) the 0.6-kbp *Pst*I/*Hin*cII fragment (a in Fig. 13), (C) the 1.6-kbp *Hin*dIII/*Sph*I fragment (b in Fig. 13), and (D) the 2.2-kbp *Cla*I/*Xba*I fragment (c in Fig. 13).

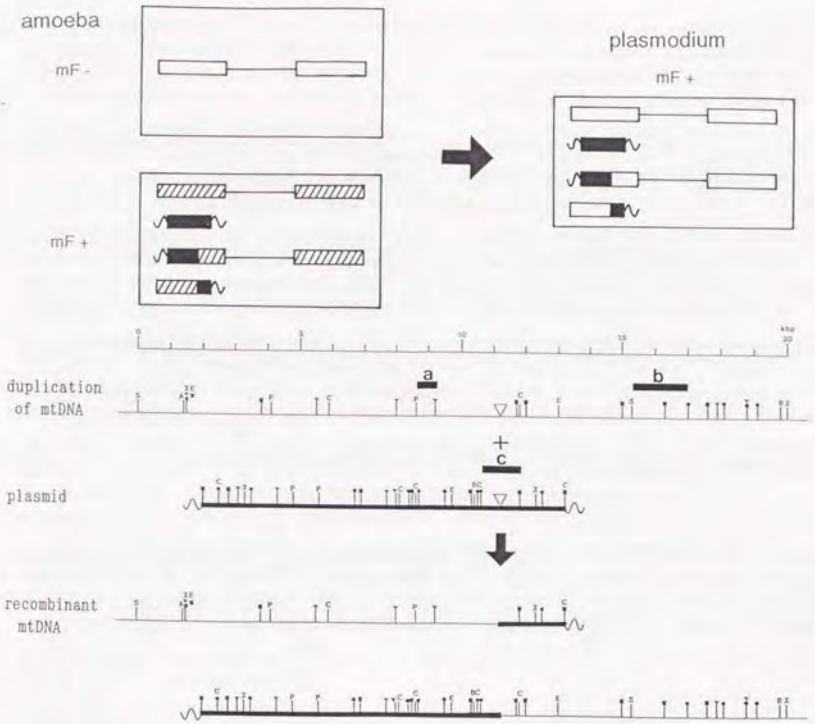

Fig. 13
(upper part) Transmission patterns of the mtDNA and the mF plasmid in the mating between the mF^+ and mF^- strains. The mtDNAs of two parents are shown by thin line (single copy region) with open or closed boxes (duplication of the mtDNA). In this mating, the recombinant mtDNA which has the mtDNA of the mF^- strain and the mF plasmid transmits to the plasmodium. (lower part) Restriction maps of a region of tandem duplication of the mtDNA of the mF^- strain, the mF plasmid and the recombinant mtDNA. The upper thin line shows the restriction map of the total length of the duplication in the mtDNA. The middle thick line shows the restriction map of the mF plasmid. The two lower lines show restriction map of the mtDNA formed by recombination between the duplication of the mtDNA and the mF plasmid. The symbols ●(*Hin*dIII), ▼ (*Hin*cII), ■ (*Eco*RV), B (*Bam*HI), C (*Cla*I), E (*Eco*RI), P (*Pst*I), A (*Sac*I), S (*Sph*I) and X (*Xba*I) indicate the various restriction sites. Thin and thick lines in the restriction map of recombinant mtDNA represent the duplication of the mtDNA and the mF plasmid, respectively. The wavy line shows the end of the mF plasmid and reflects the heterogeneity in size. Symbols a, b and c indicate regions used as probes in the experiment described in the legend to Fig. 12.

```
                                   -50                                                                                  1
MT   TTAATTATAATTTTTTTATAATGAAAAAGAACAAAAAGATAATTAATATTATGATAATATGGGTTTTACTTACAGATAGAGTAAAAGAAAGGAATATGCAA
     *  *  * *  *  *  *    * *   ** ***     *** **  *      **  *  ****-***************
P    gttttgcttaatgaatgttttcgtactttttatatgaattattttataaatgctatcaataaaagactttaaaaagagctaaa-gaaaggaatatgcaa

R1   TTAATTATAATTTTTTTATAATGAAAAAGAACAAAAAGATAATTAATATTATGATAATATGGGTTTTACTTACAGATAGAGTAAAAGAAAGGAATATGCAA

R2   gttttgcttaatgaatgttttcgtactttttatatgaattattttataaatgctatcaataaaagactttaaaaagagcTAAA-GAAAGGAATATGCAA

                                   50                                                                   100
MT   AATTACAGAAAATCTTCTTATTCAAAAAAACTACTTTATACAATATACATTCCAAAAATATCAGGAGTTACTCTACCCGAATATCCTTTAAAAGATGTTC
     *-*************************************************************************--************************
P    a-ttacagaaaatcttcttattcaaaaaaactactttatacaatatacattccaaaaatatcaggagttac--tacccgaatatccttttaaaagatgttc

R1   AATTACAGAAAATCTTCTTATTCAAAAAAACTACTTTATACAATATACATTCCAAAAATATCAGGAGTTACTCTACCCGAATATCCTTTAAAAGATGTTC

R2   A-TTACAGAAAATCTTCTTATTCAAAAAAACTACTTTATACAATATACATTCCAAAAATATCAGGAGTTAC--TACCCGAATATCCTTTAAAAGATGTTC

                                   150                                                                  200
MT   AAAAAGCTAATTTTATCCAGTTCTTTAATAGTATAGTTAAAAATGACCCAATATGGGATAATTTATGTCAAACATATAATAAAGTAAATTCTTCATTTTT
     ***********************************************************************************************************
P    aaaaagctaattttatccagttctttaatagtatagttaaaaatgacccaatatgggataatttatgtcaaacatataataaagtaaattcttcattttt

R1   AAAAAGCTAATTTTATCCAGTTCTTTAATAGTATAGTTAAAAATGACCCAATATGGGATAATTTATGTCAAACATATAATAAAGTAAATTCTTCATTTTT

R2   AAAAAGCTAATTTTATCCAGTTCTTTAATAGTATAGTTAAAAATGACCCAATATGGGATAATTTATGTCAAACATATAATAAAGTAAATTCTTCATTTTT

                                   250                                                                  300
MT   TTATCAAAAAATTAAAGATGCTTATATTAACTTAACTTTACAAACAGCTCATTATAAAGAACAAATAGCTATTTATTTATCATTTATCTTAAAATTGATT
     ***********************************************************************************************************
P    ttatcaaaaaattaaagatgcttatattaacttaacttacaaacagctcattataaagaacaaatagctatttatttatcatttatcttaaaattgatt

R1   TTATCAAAAAATTAAAGATGCTTATATTAACTTAACTTTACAAACAGCTCATTATAAAGAACAAATAGCTATTTATTTATCATTTATCTTAAAATTGATT

R2   TTATCAAAAAATTAAAGATGCTTATATTAACTTAACTTTACAAACAGCTCATTATAAAGAACAAATAGCTATTTATTTATCATTTATCTTAAAATTGATT

                                   350                                                                  400
MT   GAAATGCACCCTCAAATACCTATTAAAGATATGTGCTTAAAAGCAGTCAATTATGATGTTTATTTTGACATAAATGAGCAGGATAGTTTTTATGTTACAA
     ***********************************************************************************************************
P    gaaatgcaccctcaaatacctattaaagatatgtgcttaaaagcagtcaattatgatgtttattttgacataaatgagcaggatagtttttatgttacaa

R1   GAAATGCACCCTCAAATACCTATTAAAGATATGTGCTTAAAAGCAGTCAATTATGATGTTTATTTTGACATAAATGAGCAGGATAGTTTTTATGTTACAA

R2   GAAATGCACCCTCAAATACCTATTAAAGATATGTGCTTAAAAGCAGTCAATTATGATGTTTATTTTGACATAAATGAGCAGGATAGTTTTTATGTTACAA

                                   450                                 479                                500
MT   TAAAACAGAATCCTTATAGTCCTATTGAAATTAGTCTTCAACATGATAATATTTGTTTGCACACTTTCCATTTATTACTTATTGAAGAAATATTTCTACA
     ************************************************************* *   * *  **   *   **  *     * *     * *
P    taaaacagaatccttatagtcctattgaaattagtcttcaacatgataatatttgtttgaaagatataaatcgctatattgctctattagttcaaatgga

R1   TAAAACAGAATCCTTATAGTCCTATTGAAATTAGTCTTCAACATGATAATATTTGTTTGaaagatataaatcgctatattgctctattagttcaaatgga

R2   TAAAACAGAATCCTTATAGTCCTATTGAAATTAGTCTTCAACATGATAATATTTGTTTGCACACTTTCCATTTATTACTTATTGAAGAAATATTTCTACA

                                   559
MT   AAACTTTCAAGATAATATTGAACCATTTTCTGATCTCTT
     ***   *    ** *  *   *  *  *     * *  *
P    aaaacatttttatgaattattagaaagtatatttattat

R1   aaaacatttttatgaattattagaaagtatatttattat

R2   AAACTTTCAAGATAATATTGAACCATTTTCTGATCTCTT
```

Fig. 14
Comparison of sequences between the mtDNA, the mF plasmid and the recombinant mtDNA. Sequences are numbered relative to the first nucleotide of the so-called identical sequence of the mtDNA, and matches between the main mtDNA (capitals) and the mF plasmid (small letters) are indicated by asterisks. Sequences are shown from -80 to 559. Recombinant molecules are classified into two types: R1 consists of the minus region of the mtDNA (- 0), the so-called identical region (1 - 479) of the mtDNA type and the plus region of the mF plasmid (480 -); and R2 consists of the minus region of the mF plasmid (- 0), the so-called identical region (1 - 479) of the plasmid type and the plus region of the mtDNA (480 -). The so-called identical region in the recombinant mtDNAs (capitals) is underlined. The deletions of nucleotides in the mF plasmid and the recombinant mtDNA are shown by hyphens (-).

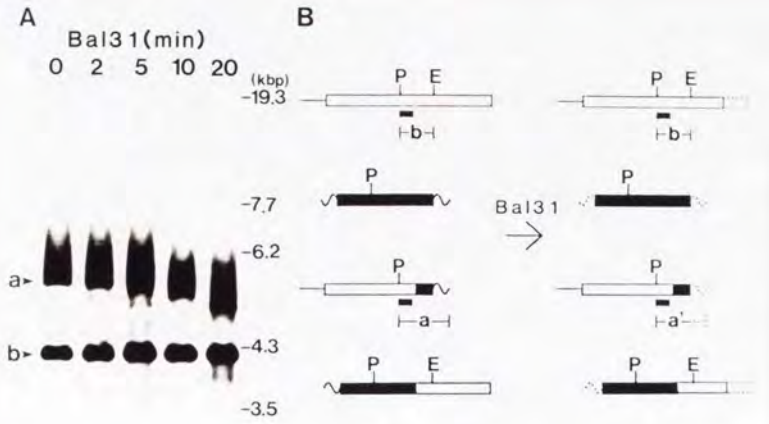

Fig. 15
Sensitivity to *Bal*31 exonuclease of the end of the recombinant mtDNA with the mF plasmid. Total DNA from isolated mitochondria was digested with *Bal*31 for 0, 2, 5, 10 and 20 min, as indicated. The samples were then digested with *Pst*I and *Eco*RI, and digests were fractionated by electrophoresis and blotted. The membrane was hybridized with the 0.6-kbp *Pst*I/*Eco*RI fragment (a in Fig. 13; closed box in the diagram). The representation of the experiment in the case of the recombinant mtDNA from the right duplication is shown on the right (B). The thick line shows the single-copy region of mtDNA after the duplication. The open bar and closed bar show the duplication of mtDNA and the mF plasmid, respectively. The wavy line shows the end of the mF plasmid. The restriction sites of *Pst*I and *Eco*RI are shown by P and E, respectively. The dotted bar and dotted wavy line show the regions that are digested with *Bal*31. In the duplication that had not crossed over with the mF plasmid, the probe hybridized to the 4.2-kbp *Pst*I/*Eco*RI restriction fragment (b). In the recombinant mtDNA that had crossed over with the mF plasmid, the probe hybridized to the *Pst*I restriction fragment, which included the right end of the mF plasmid, so that the hybridization pattern is browed at approximately 5.3 kbp (a). The 4.2-kbp *Pst*I/*Eco*RI restriction fragment (b) was not digested with *Bal*31, because it is an internal fragment that is unaffected by *Bal*31. However, the *Pst*I fragment, including the right end of the mF plasmid (a), was digested from the end so that it migrated more rapidly than the undigested fragment (a').

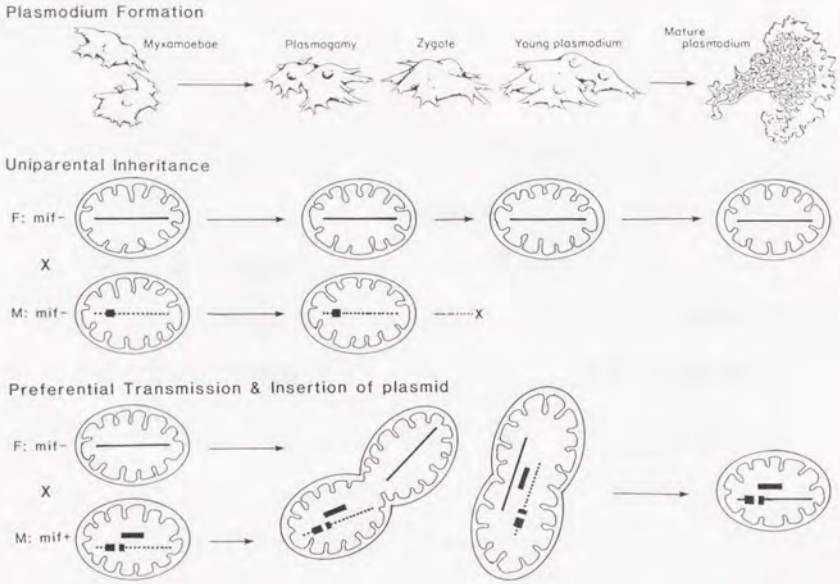

Fig. 16
The behavior of mitochondria, mtDNA and mF plasmid during plasmodium formation. The thin and dotted lines represent the mtDNA in individual mitochondria of two parents. The mF plasmid is represent by the closed box. A mating between mF^- strains results in uniparental inheritance of the mtDNA. In matings between mF^+ and mF^- strains, mitochondrial fusion occurs. In fused mitochondrion, a recombination occurs between the mtDNA and the mF plasmid.

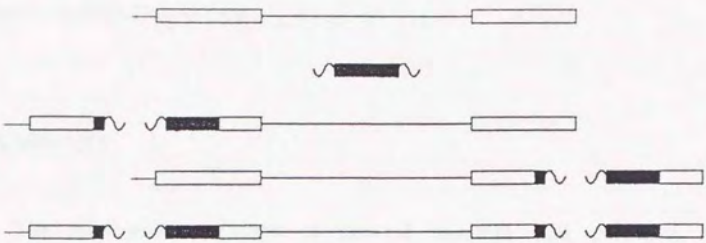

Fig. 17
Representation of the diversity of the recombinant mtDNAs. The duplication and the single-copy region of the mtDNA are shown by open boxes and thin lines, respectively. The mF plasmid is shown by closed boxes and the terminal repeats at the ends are shown by wavy lines. The lower three lines show the recombinant mtDNAs that have crossed over at the left duplication, at the right duplication, and at both duplications (from top to bottom).

CHAPTER III

Genetic organization of mF plasmid that promotes the mitochondrial fusion in *P. polycephalum*

SUMMARY

The nucleotide sequence of the mF plasmid that promotes the mitochondrial fusion in *P. polycephalum* was determined. It was 14503 bp in size, and contains 10 ORFs. All ORFs except one were encoded on the same DNA strand. The numbers of amino acid residues of putative proteins derived from 9 ORFs on the same strand were 231, 163, 640, 235, 118, 1130, 366, 309, and 547. The amino-acid sequences of ORFs identified in this CHAPTER did not show significant homology to any amino-acid sequences in the databases. A brief transcription map of the mF plasmid was created using the cloned restriction fragments of the mF plasmid. It suggested that the transcription initiation site located near the inner end of the left TIR of 591 bp and that the transcripts of 1.0, 3.4 and 4.6 knt and longer transcripts started from this putative transcription initiation site. The 3.5-knt transcripts corresponded to the coding region of the ORF-1130 may be derived from this longer transcripts. Southern hybridization using ^{32}p-labelled mtRNA showed that the transcripts on the upstream region of the mF plasmid was about 500 times more in quantity than that of the lower region.

INTRODUCTION

In the true slime mould. *P. polycephalum*, the mF plasmid is apparently responsible for the promotion of mitochondrial fusion. In the mF plasmid, the terminal regions have been sequenced to determine the structural features of the plasmid's ends (CHAPTER I). The characteristic features of its ends were three TIRs; a 205-bp TIR at extreme ends, a 591-bp TIR at inner region and a 144-bp repeating unit which exists between them. In the right terminal region, there was ORF-547 which had extensive homology with polymerization domain of the putative DNA polymerases. Another sequenced region of the mF plasmid was the 475-bp almost identical sequence with the mtDNA (CHAPTER II). Following the mitochondrial fusion in zygote, the recombination occurred between this identical sequences of the mtDNA and of the mF plasmid, and recombinant mtDNA was generated with the ends of the mF plasmid as its ends.

Linear mitochondrial plasmids have been detected in a wide variety of higher plants and fungi (for reviews, see Samac and Leong 1989; Meinhardt *et al.* 1990). The complete nucleotide sequences of some linear mitochondrial plasmids have been determined, such as S1 (6397 bp) and S2 (5452 bp) in maize (Paillard *et al.* 1985, Levings and Sederoff 1983), pCIK1 (6752 bp) in *C. purpurea* (Oeser and Tudzynski 1989), pMC3-2 (6044 bp) in *M. conica* (Rohe *et al.* 1991), *kalilo* (8643 bp) in *N. intermedia* (Chan *et al.* 1991), *maranhar* (7052 bp) in *N. crassa* (Court and Bertrand 1992) and pAL2-1 (8395 bp) in *P. anserina* (Hermanns and Osiewacz 1992). They encode DNA and/or RNA polymerase and few short ORFs as follows: DNA polymerase and 2 short ORFs on S1, RNA polymerase and a short ORF on S2, DNA and RNA

polymerases and 4 short ORFs on pCIK1, DNA polymerase and a short ORF on pMC3-2, DNA and RNA polymerases on *kalilo*, DNA and RNA polymerases and 3 short ORFs on *maranhar*, and DNA and RNA polymerases on pAL2-1. Although these linear plasmids have been sequenced, knowledge about their function is still rather poor. It is known that the senescences in *N. intermedia* and *N. crassa* is induced by the integrations of *kalilo* and *maranhar* plasmids into the mtDNAs, respectively. However, *kalilo* and *maranhar* plasmids encode only DNA and RNA polymerases, and do not encode any genes which cause senescence.

The genetic organization of the mF plasmid is interesting because it is the longest one among the mitochondrial linear plasmids and causes the mitochondrial fusion. To detect ORFs of the mF plasmid and to determined gene(s) associated with the mitochondrial fusion, the total DNA sequence of the mF plasmid was determined.

MATERIALS AND METHODS

Strains and culture conditions were described in MATERIALS AND METHODS of CHAPTER I. Sequence was carried out according to the methods in CHAPTER I.

For Northern hybridization, 10 μg of mtRNA was separated through agarose gels after denaturation with glyoxal as described by Maniatis et al. (1982). The RNA gels were blotted on a nylon membrane with the vacuum blotting system (Pharmacia LKB Biotechnology, Sweden) as described by Takano *et al.* (1990). Filters were prehybridized and hybridized as described by Maniatis *et al.* (1982).

To analyze the difference of the abundance of the transcripts, five probes were chosen: a (nt1378-2116), b (nt4340-5083), c (nt6170-6803),

d (nt8954-6968) and e (nt12034-12838). The fragments of these probes were generated from the cloned restriction fragments. The fragments were subject to electrophoresis, stained with ethidium bromide, and then blotted on a nylon membrane. The volumes of the fragments were changed. The mtRNA from the isolated mitochondria was labelled with ^{32}P by the method of Sakai et al. (1981). The prehybridization, hybridization and washing were performed according to the method of Sakai *et al.* (1991).

RESULTS

Nucleotide sequence and coding capacity of the mF plasmid

To detect what ORFs exist on the mF plasmid, the complete nucleotide sequence of the mF plasmid was determined (Fig. 18). In the previous study, it was obvious that the ends of the mF plasmid had three TIRs. Since the number of 144-bp repeating units was different each other, the selected clones for the determination of the nucleotide sequences of the left and right ends influenced the size of the mF plasmid. If the nucleotide sequences of the clones, NPT4X-34 and -64 in CHAPTER I were used for the nucleotide sequences of the ends, the mF plasmid was 14503 bp in length (Fig. 18). Overall, the mF plasmid had an AT-content of 75.0%.

A search for ORFs was performed by translating the sequences in all reading frames with the universal genetic code. The complete sequence of the mF plasmid contains 10 ORFs which had potential methionine initiation codons (Figs. 18 and 19). These ORFs were named based on the number of the amino-acid residues. All ORFs except one (ORF-221) were encoded on the same strand, named coding strand. The order of ORFs on

the coding strand was ORF-231 (nt1577-2272), ORF-163 (nt2269-2760), ORF-640 (nt2821-4743), ORF-235 (nt4849-5556), ORF-118 (nt5726-6082), ORF-1130 (nt6145-9537), ORF-366 (nt9616-10716), ORF-309 (nt10685-11614) and ORF-547 (nt11753-13396) from the upper region to the lower region. One ORF on the other strand was ORF-221 (nt1485-780) reported in CHAPTER I. At two locations, two ORFs overlapped: ORF-231 and ORF163 (nt2269-2272); ORF-366 and ORF-309 (nt10685-10716). Moreover, ORF-366 overlapped on the identical sequence with the mtDNA (Fig. 19; CHAPTER II). Except ORF-547, the amino-acid sequences derived from these ORFs did not show significant homology to any amino-acid sequences in the data base (see, DISCUSSION).

Transcription map

The cloned fragments of the mF plasmid have been used as probes to produce a brief transcription map of the mF plasmid. The fragments representing 11 different regions of the mF plasmid (1-11 in Fig. 20), were hybridized with immobilized mtRNA from isolated mitochondria. The mtRNA from the plasmid-free strain gave no hybridization signal with any of these 11 probes used, providing that these transcripts are indeed plasmid-derived. When the probes 1 and 11 were used, there were no major detectable transcripts, suggesting very low concentrations of the transcripts in the regions of TIRs. When upper region of the coding strand (regions 2-5) was used as probes, major transcripts of 1.0, 3.4 and 4.6 knt could be detected (Fig. 20), and in addition there are the minor signals of over 4.6 knt in the long-exposed autoradiograms. The probe 2 hybridized to specific RNA species: 1.0 knt, 3.4 knt and 4.6 knt. The probes 3 and 4 hybridized to 3.4- and 4.6-knt RNA species and the probe 5 only hybridized to 4.6 knt. By these hybridization patterns and the

lengths of these transcripts, it was suggested that the transcription initiation site mapped near the inner end of the left TIR of 591 bp and that transcripts started at the left end and went to the lower region on the coding strand (Fig. 21).

When the probes 6-8 which are parts of the coding sequence of ORF-1130 were used, the 3.5-knt transcript and the longer transcripts of about 6-8 knt were only detected in the long-exposed autoradiogram (Fig. 20). The hybridization patterns of probes 2-8 suggested that these longer transcripts started at the left end and went through the region 2-8. The 3.5-knt transcript may be generated from these longer transcripts by splicing of RNA. With the probes 9-10, very low abundance of transcripts were observed, however, these regions were also transcribed (Fig. 22). The schema of the transcription of the mF plasmid is shown in Fig. 21.

The Northern hybridization suggested that the abundance of the transcripts were different between the 5' upper region and the lower region of the coding strand. Thus, the variance of the abundance of the transcripts was analyzed (Fig. 22). The results showed that the mtRNA products of region (a) is about 50 times more in quantity than that of region (c). In the autoradiogram with the long exposure, moreover, the mtRNA products of region (c) is about 10 times more than that of regions (d) and (e). Therefore, mtRNA hybridized with region (a) may be produced about 500 times more than that of (d) and (e).

DISCUSSION

Nucleotide sequence and coding capacity of the mF plasmid

The mF plasmid was 14503 bp in size. The complete nucleotide sequences have been determined on some linear mitochondrial plasmids.

The mF plasmid was the largest in length among not only the sequenced mitochondrial plasmids but also the unsequenced mitochondrial plasmids.

The mF plasmid contained 10 ORFs. The complete sequence of pClk1 contains two long ORFs and four smaller ORFs, and it has the most number of coding sequences among the known plasmids before this study. The known plasmids including pClK1 have only the coding sequence of DNA polymerase and/or RNA polymerase. The mF plasmid also had the coding sequence of DNA polymerase (ORF-547; see CHAPTER I). Moreover, it had 8 ORFs. It seems to be reasonable that the mF plasmid had more number of ORFs than other linear mitochondrial plasmids, because the mF plasmid causes the mitochondrial fusion. However, other ORFs in the mF plasmid showed no homology to the known amino-acid sequences in the database. The computer analysis which was constructed by Kyte and Doolittle (1982) predicts membrane-associated proteins. The ORFs in the mF plasmid were not predicted to be membrane proteins by this analysis.

While the insensitive to λ exonuclease suggests that the 5' ends of the mF plasmid are protected by terminal proteins (CHAPTER I), the nucleotide sequence did not disclose a distinct gene that might code for a terminal protein. Chan *et al.* (1991) and Court *et al.* (1992) have suggested that the cryptic amino-terminal domains which precede the exonuclease domains of the plasmid DNA polymerases may be parts of the terminal proteins. Within these domains, there are two SYKN sequence motifs which are comprised of consistently spaced serine, tyrosine, lysine and asparagine residues and they are also present in the terminal proteins of bacteriophage φ29 and PRD1 and the linear adenovirus Ad2. These sequence motifs existed in the amino-acid sequence derived from ORF-309 locating just before ORF-547 (nt11110-11161 and nt11255-11318).

However, the weakness in this analysis is that there was no information about the amino-acid sequence of even one terminal protein from a mitochondrial plasmid; nor there is any structural or genetic data to support the notion that the SYKN motif is relevant to the function of any known terminal protein. Much more research is needed to further define the terminal proteins of the linear mitochondrial plasmids including the mF plasmid.

Transcription map

The transcription initiation site may locate near the inner end of the left TIR of 591 bp and that the transcripts of 1.0, 3.4 and 4.6 knt and longer transcripts started from this putative transcription initiation site. The 3.5-knt transcripts corresponded to the coding region of ORF-1130 might derived from the longer transcripts of over 8 knt. The Southern hybridization using ^{32}p-labelled mtRNA showed that the transcripts of the upper region on coding strand were about 500 times more in quantity than that of the lower region. The transcriptions of the ORFs of pClK1 and *kalilo*, have been analyzed (Düvell *et al.* 1988; Gessner-Ulrich *et al.* 1991; Griffiths 1992). In the case of pClK1, two major transcripts have been detected, corresponding to ORF1 (DNA polymerase) and ORF2 (RNA polymerase). These ORFs start in the TIRs of pClK1 and go through the inner region. In pClK1, it has been established that both transcripts start at the end of the TIR. The concentrations of two major transcripts are almost equivalent. The same transcriptional manner is used in *kalilo*. The mode of transcription of the mF plasmid was more complex than that of other mitochondrial plasmids and quite different from that of other linear mitochondrial plasmids. Further work is needed to determined the precise starting and terminating points of transcripts.

Fig. 18

Complete nucleotide sequence of one of the two strands of the linear DNA of the mF plasmid. The amino-acid sequences of the putative proteins encoded by 9 ORFs on the coding strand are shown below the nucleotide sequence. The amino-acid sequence of ORF-221 on the other strand is shown above the nucleotide sequence. The stop codons are represented by asterisks. The numbers of start and last nucleotides of ORFs are given in the text.

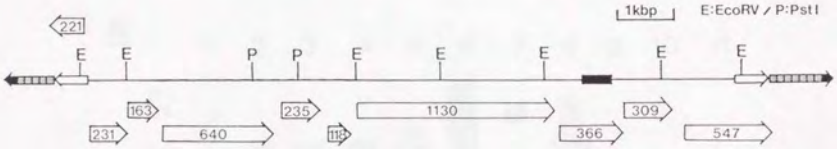

Fig. 19
Positions of ORFs of the mF plasmid. The locations and orientations of ORFs are indicated by open arrows. The numbers in open arrows show the numbers of amino-acid residues of ORFs. To facilitate the positions of ORFs, the restriction map of the mF plasmid is added, including *Eco*RV (E) and *Pst*I (P) sites. Three TIRs are shown by the same symbols as indicated in Fig. 8. The identical sequence with the mtDNA is shown by closed box in the map.

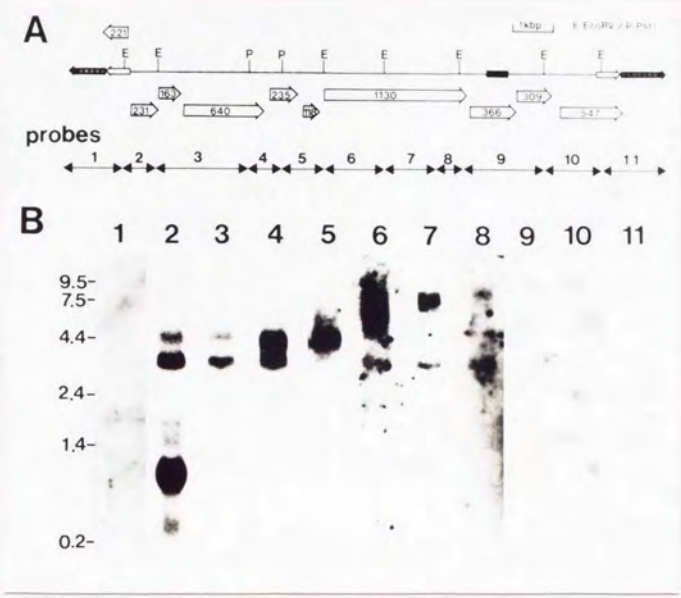

Fig. 20
Northern analysis of mtRNA from the mF^+ strain. A) The schematic representation of the locations and orientations of ORFs. The characters and symbols in the map are the same as those in Fig. 19. The regions of probes are indicated below the map. B) The hybridization patterns with each probe.

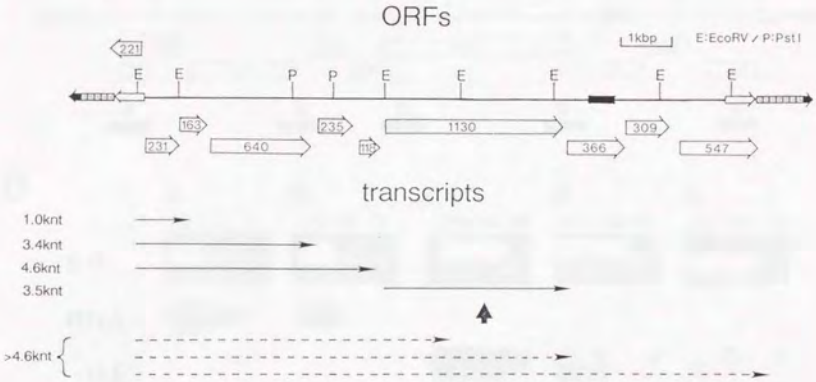

Fig. 21
The brief transcription map of the mF plasmid derived from hybridization data in Fig. 20. The characters and symbols in the map are the same as those in Fig. 19. The locations and putative orientations of transcripts are shown by arrows below the map. The sizes of transcripts are indicated on the left side. The longer transcripts of low concentration are indicated by dotted arrows. The 3.5-knt transcript may be derived from this longer transcripts.

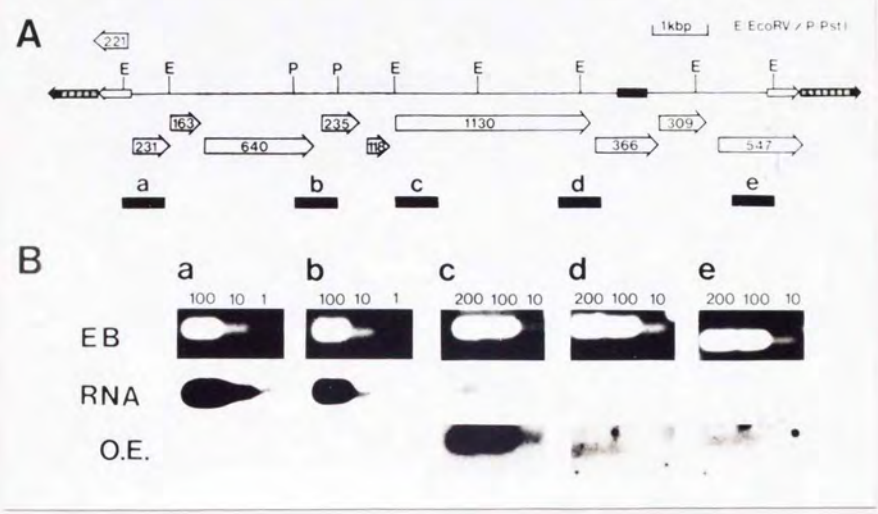

Fig. 22
A) The characters and symbols in the map are the same as those in Fig. 19. The regions of probes are shown below the map. B) The fragments of these regions were generated from the cloned fragments, then electrophoresed. The panels of EB show the patterns of staining with ethidium bromide. The relative amount of samples are shown above the lanes. The panels of RNA show the hybridization patterns with labelled mtRNA. The panels of O.E. show the hybridization patterns of the regions c, d and e in a long-exposed autoradiogram.

CHAPTER IV

Genetic organization of the mF plasmid in the mitochondrial fusion-deficient strains isolated from the mitochondrial fusion strains in *P. polycephalum*

SUMMARY

The mF plasmid causes the mitochondrial fusion during plasmodium formation and during sporulation. By the fluorescence-microscopic observations, the mitochondrial fusion-deficient strains (Δmif$^-$ strains) have been isolated from the mitochondrial fusion strains which have the mF plasmid. To detect gene(s) associated with the mitochondrial fusion, the mF plasmid in the Δmif$^-$ strains was analyzed. The results showed that the region near the identical sequence between the mtDNA and the mF plasmid was deleted in all Δmif$^-$ strains. The organization of the mitochondrial genome in one of these Δmif$^-$ strains suggested that the 2.2-kbp region including the identical sequence was deleted by the recombination at 12-bp sequences. This deletion caused the deletion of the coding sequences of ORF-1130 and -366. The slot hybridization of mtRNA showed that the abundance of the mtRNA of the Δmif$^-$ strain decreased to the same volume as that of the mif$^-$ strain which has no mitochondrial plasmid. These results suggested that the deleted region was important not only for the transcription of the mF plasmid but also for the mitochondrial fusion.

INTRODUCTION

A linear mitochondrial plasmid of about 16 kbp, named mF, causes mitochondrial fusion in zygote and during sporulation in *P. polycephalum*. In matings between the mif$^-$ and mif$^+$ strains, the small mitochondria fuse with one another to form large multinucleate mitochondria. Following the mitochondrial and mitochondrial-nuclear fusions in zygote, the recombination occurred between the mtDNA and the mF plasmid, and recombinant mtDNA was generated with the ends of the mF plasmid as its ends (CHAPTER II). In CHAPTER III, I showed that the mF plasmid had 10 ORFs and that these ORFs were transcribed. However, except ORF-547 which coded the DNA polymerase, the computer analysis of these ORFs gave no cues for their function.

There were many strains including mitochondrial fusion (mif$^+$) strains (Kawano *et al.* 1991b, 1993). While almost of the mitochondrial fusion-deficient strains have no mitochondrial plasmid, some mitochondrial fusion-deficient strains have been isolated from the mitochondrial fusion strains. Thus, the mitochondrial fusion-deficient strains are classified to two types: mif$^-$ strains which do not carry the mF plasmid and the Δmif$^-$ strains which isolated from the mitochondrial fusion strains carrying the mF plasmid. The defect of the mitochondrial fusion in the Δmif$^-$ strains may be cause by defect of the gene(s) on the mF plasmid. To detect alterations in the mF plasmid and the mtDNA in the Δmif$^-$ strains, the mitochondrial genomes of the Δmif$^-$ strains have been analyzed.

MATERIALS AND METHODS

The amoebal strains of *P. polycephalum*, CH934, CH938, RA221 and

AI16 are derivatives of Colonia (Youngman *et al.* 1981). The strains, NG6, NG7, NG11, NG15, NG19 and NG327 are derivatives of Ng (Kawano *et al.* 1991a, 1993). The strain RN68 is progeny from the crossing between Colonia derivative and Ng derivative. The strains HI10 and HI14 are isolated from strain Hi (Kawano *et al.* 1993). The strains DP12, DP13, DP14, DP15, DP74, DP75 and DP89 were obtained from D. Pallotta (Kirouac-Brunet *et al.* 1981). The strain CB8 was isolated from strain Cb (Kawano *et al.* 1987). The strain LU887 was obtained from R. W. Anderson (Anderson 1977). The strain OS57 was obtained from N. Kamiya (Kawano *et al.* 1987).

Mitochondria, total DNA from the isolated mitochondria and the mF plasmid were isolated by the methods of Kawano *et al.* (1991a). Southern hybridization was done by the method of Takano *et al.* 1990). Construction of the restriction map of the mitochondrial genomes of the Δmif$^-$ strains is performed as described previously (Takano *et al.* 1990). Sequence was carried out according to the method in CHAPTER I.

The mtRNAs were isolated from mif$^+$ (CH934 x NG7), Δmif$^-$ (AI16 and NG15) and mif$^-$ (CH934 x CH938) strains for slot hybridization. The amounts of slotted mtRNAs are 10 mg, 1 mg, 100 ng and 10 ng for lane 1, 2, 3 and 4 in Fig. 27, respectively. To analyze the abundance of the transcripts in the Δmif$^-$ strain, two regions were chosen: a (nt1378-2116) and b (nt6170-6803). Slot blotting, labelling of DNA probes, hybridization and washing of the membranes were performed according to the method described by Sakai *et al.* (1991).

RESULTS

The mitochondrial genomes of the mif$^+$, Δmif$^-$ and mif$^-$ strains were analyzed by Southern hybridization (Fig. 23). I chose the six mif$^+$ strains,

six Δmif⁻ strains and five mif⁻ strains. The total DNAs from isolated mitochondria from these strains were digested with *Eco*RV and *Pst*I, subject to electrophoresis and then blotted on a nylon membrane. With the cloned fragments of all areas of the mF plasmid as probes, Southern hybridizations were done to detect the heterogeneity of the mF plasmid among the strains. While these probes ordinally hybridized to the same fragments of the mitochondrial genomes of the mif⁺ and Δmif⁻ strains, they did not hybridized to any fragments of the mitochondrial genomes of the mif⁻ strains. However, the probe (b) in Fig. 23 including the identical sequence with the mtDNA hybridized to the restriction fragments of the mitochondrial genomes of the mif⁻ strains in spite of the length variation of fragments among the strains. This showed that the identical sequence existed in all strains which do not carrying the mF plasmid. Between the mif⁺ and Δmif⁻ strains, the difference of the hybridization patterns was generated with the probe (c) in Fig. 23. While the 1.7-kbp *Eco*RV/*Pst*I fragment was detected in the mif⁺ strains, the 1.4-kbp fragment was hybridized in the Δmif⁻ strain (lane 7-10 in Fig. 23a-c). Two Δmif⁻ strains (lane 11 and 12 in Fig. 23a-c) did not hybridized to this fragment, suggesting the loss of this region. The schematic representation of the lost or deleted regions of the mF plasmid in the Δmif⁻ strains is shown in Fig. 24. In the plasmodial strain, NG11 x NG19, the deleted mF plasmid coexisted with the normal mF plasmid, and the mitochondrial fusion occurred in this strain. In all Δmif⁻ strains, the region near the recombination site was deleted.

In one plasmodial strain, AI16 x NG15, the deletion of the mF plasmid occurred at only one region. Thus, the mitochondrial genome of this strain was analyzed. The fine restriction map of the mitochondrial genome of AI16 x NG15 was constructed (Fig. 25). In this mitochondrial

genome, there was only the recombinant mtDNA and the un-recombined mtDNA and the free mF plasmid were lost. The 2.2-kbp deletion occurred mainly on the mF plasmid region of the recombinant mtDNA (Fig. 25). This region included the identical sequence with the mtDNA and small regions of main mtDNA, too. This was due to the deletion of the coding sequence of ORF-1130 and -366. The deletion of ORF-1130 created new ORF (ORF-800; 800 amino-acid residues) which had N-terminus region of ORF-1130. The sequence at the deleting point in the Δmif^- strain showed that the 12 nucleotide sequences, 5' TATTGAAACATT 3' and 5' TATTGAACCATT 3' existed on the regions of the mF plasmid (nt8458-8457 on the sequence of the mF plasmid; Fig. 18) and the mtDNA region of the recombinant mtDNA, respectively (Fig. 26). The recombination occurred at these 12-bp sequences and caused deletion of the 2.2-kbp sequence.

The abundances of the mtRNA of the coding sequences on the mF plasmid in the deleted recombinant mtDNA were analyzed by the slot hybridization (Fig. 27). The total mtRNAs were isolated from the mif^+ strain (CH934 x NG7), the Δmif^- strain (AI16 x NG15) and the mif^- strain (CH934 x CH938), then blotted on a nylon membrane. The regions a and b in Fig. 27 were used as probes. When the probe (a) was used, the abundance of the transcripts in the Δmif^- strain decreased at least 1000 times lesser than that of the mif^+ strain. With the probe (b), the slot hybridization gave the same results. These results showed that the abundance of the transcripts of the Δmif^- strain decreased to the value of the mif^- strain which has no mitochondrial plasmid. In the Northern hybridization experiments, the probes of the mF plasmid (probes 1-11 in Fig. 20) gave no hybridization signals to the mtRNA of the Δmif^- strain, confirming the decease of the abundance of the transcripts in the Δmif^-

strain. These results suggested that the deleted region of the mF plasmid was important not only for the transcription of the mF plasmid but also for the mitochondrial fusion.

DISCUSSION

Six Δmif^- strains have been isolated from the mif^+ strains. The restriction map of the one of the Δmif^- strain, AI16 x NG15, showed that the 2.2-kbp deletion including the identical sequence with the mtDNA occurred in this strain. In other Δmif^- strain, the probe c in Fig. 23 hybridized to 1.4-kbp band similar to the band of AI16 x NG15. It suggested that the same deletion occurred on these strains. In the present time, other types of Δmif^- strains did not isolated. If the mif^+ strains which has the deleted mF plasmid were selected, they are useful strains. Unfortunately, these strains were not isolated. In the Δmif^- strains used here, the un-recombinant mtDNA and the free plasmid does not exist. The relationship between the deletion of the mF plasmid and the loss of the free plasmid remains to be resolved. It is a cue that the recombinant mtDNA in Δmif^- strains has only one copy of the identical sequence between the mtDNA and the mF plasmid. However, in the present, the mechanism of the loss of the free plasmid is unknown.

The results showed that the region which deleted in the Δmif^- strains is important not only the transcription but also the mitochondrial fusion. It is a open question whether ORF-1130 and -366 directly act for the mitochondrial fusion or not. It is possible that the deficient of the mitochondrial fusion is due to the decrease of the abundance of the mtRNA of ORF-1130 and -366. Moreover, the relationship between the loss of the free plasmid and the deficient of the mitochondrial fusion also remains to investigated.

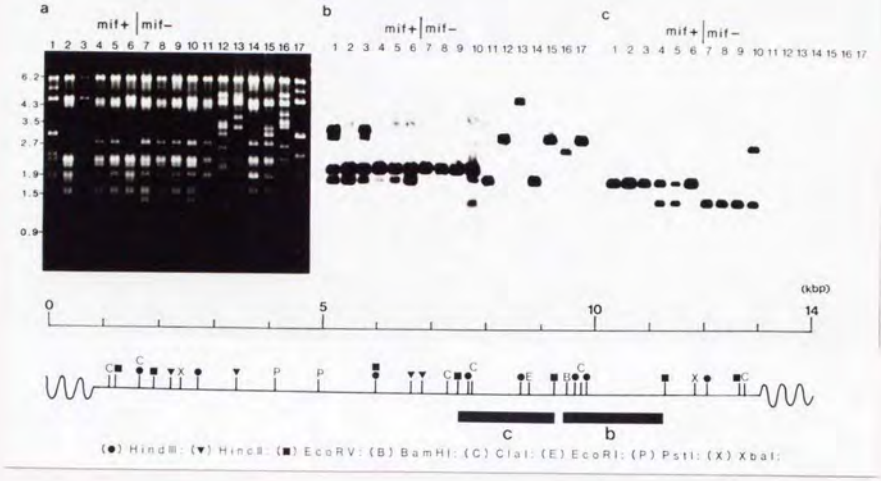

Fig. 23
The total DNAs of isolated mitochondria from 17 plasmodial strains were digested with *Eco*RV and *Pst*I, and subject to electrophoresis. Panels show the patterns of staining with ethidium bromide (a); the hybridization patterns with probes (b) and (c) in the map. The characters and symbols in the map are the same as those in Fig. 4 . The strains used were, 1 (RA226 x NR96), 2 (AI16 x NG7), 3 (RA221 x RN68), 4 (NG11 x NG19), 5 (AI16 x NG327), 6 (NG6 x NG7), 7 (AI16 x NG19), 8 (AI16 x NG15), 9 (AI16 x HI10), 10 (AI16 x HI14), 11 (AI16 x CB8), 12 (DP12 x DP13), 13 (DP14 x DP15), 14 (DP75 x DP74), 15 (DP89 x LU887), 16 (AI16 x OS57), 17 (CH934 x CH938). The strains 1-6 are the mif^+ strains. The strains 7-12 are the Δmif^- strains. The strains 13-17 are mif^- strains.

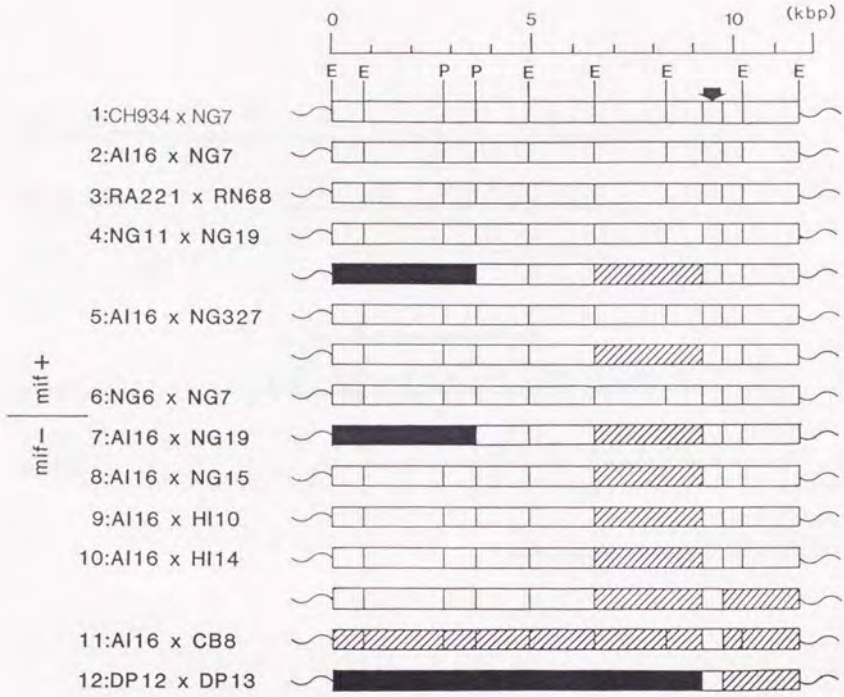

Fig. 24
The organizations of the mF plasmid in 12 plasmodial strains. The number of the strains corresponds to that in Fig. 23. Above the schematic map of the mF plasmid from RA226 X NR96, the *Eco*RV and *Pst*I sites are indicated by E and P, respectively. The regions which are shown by closed boxes are completely deleted. The regions which are shown by hatched boxes were partially deleted or regions of very low concentration. The identical sequence with the mtDNA is indicated by an arrow. Terminal repeats are shown by wavy lines.

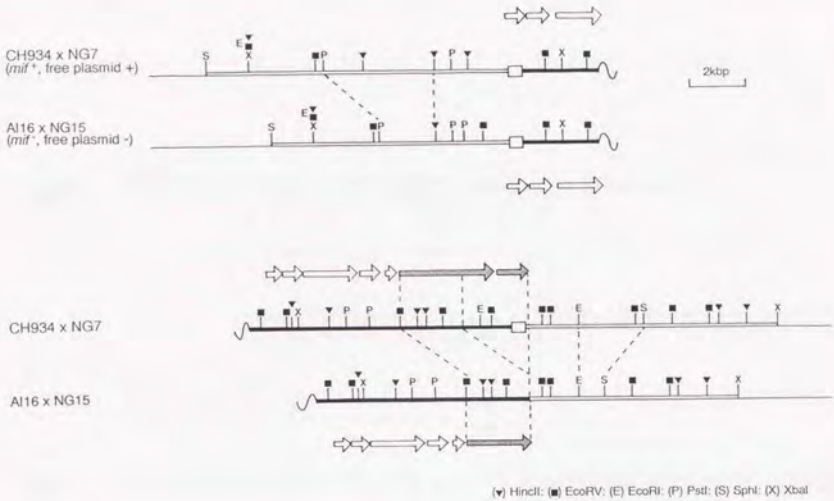

Fig. 25
The mitochondrial genome from the Δmif⁻ strain, AI16 x NG15 is compared with the recombinant mtDNA from the mif⁺ strain, CH934 x NG7. The appropriate corespondent sites between the mif⁺ strain and the Δmif⁻ strain connect by dotted lines. The symbols in the map are the same as those in Fig. 13. Thin line, open bar, open square, closed bar and wavy line show single-copy region of the mtDNA, duplication of the mtDNA, identical sequence between the mF plasmid and the mtDNA, and terminal region of the mF plasmid, respectively. ORFs on the coding strand of the mF plasmid are shown by arrows above the map. ORFs which were deleted in the Δmif⁻ strain were hatched.

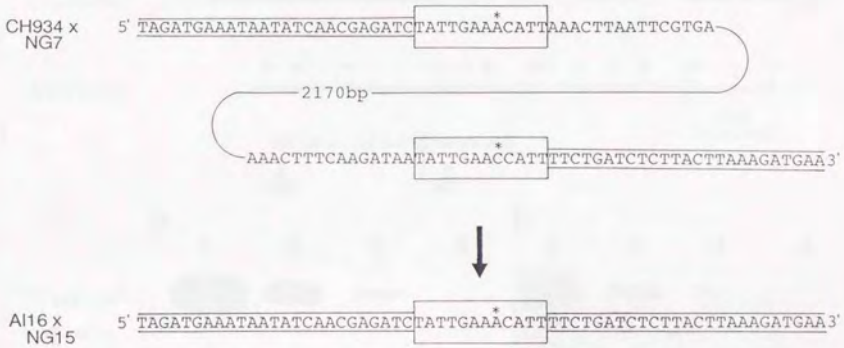

Fig. 26
The nucleotide sequence at the deletion point in the Δmif⁻ strain is compared with that of the mif⁺ strain. The recombination occurred between the 12-bp sequences in boxes of the mif⁺ strain and generated the 2.2-kbp deletion in the Δmif⁻ strain. The different nucleotide between these 12-bp sequences is represented by an asterisk.

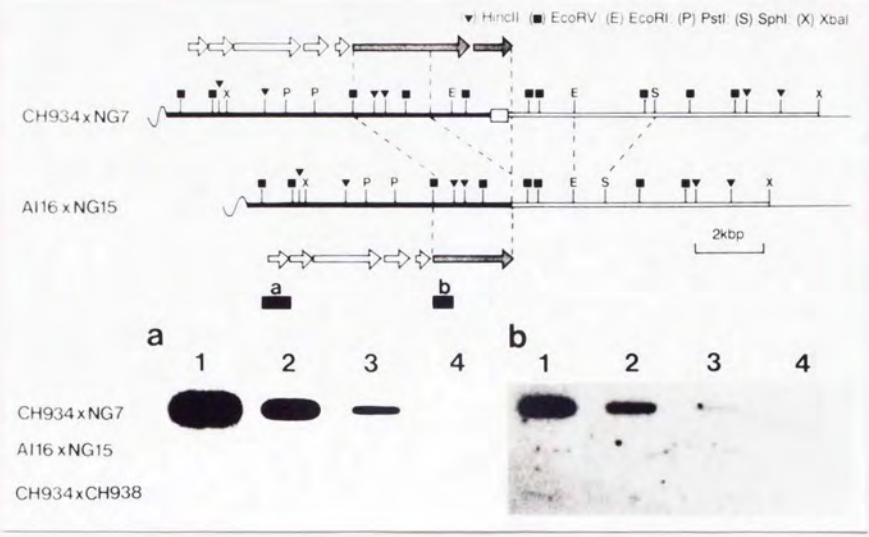

Fig. 27
The decrease of the transcripts in the Δmif⁻ strain. The upper diagram is the same one in Fig. 25. The mtRNA of CH934 x NG7 (mif⁺), AI16 x NG15 (Δmif⁻) and CH934 x CH938 (mif⁻) were isolated and blotted. The volume of slotted mtRNA are 10 μg, 1μg, 100 ng and 10 ng for lane 1, 2, 3 and 4, respectively. The regions of probes (a) and (b) are represent in the upper map.

CONCLUSION AND PERSPECTIVE

The results obtained in the present study can be summarized as follows (Fig. 28). (1) The mF plasmid was a linear molecule with three TIRs including the repeating units. (2) In matings between mF^+ and mF^- strains, the reconstruction of the mitochondrial genome was occurred by the recombination between the mtDNA and the mF plasmid. This recombination was due to reciprocal crossing-over at the identical sequences of the mtDNA and of the mF plasmid. (3) The mF plasmid had 10 ORFs including the putative DNA polymerase. The transcription initiation site might locate near the inner end of the left TIR of 591 bp and the transcripts on the upstream region of the mF plasmid was about 500 times more in quality than the lower region. (4) In all Δmif^- strains which have been isolated from the mitochondrial fusion strains, the region near the identical sequence was deleted. The deletion caused the deletion of the coding sequences of ORF-1130 and -366, and the decrease of the abundance of the RNA of the mF plasmid.

This is the first study that the recombination of the mitochondrial genome is apparently associated with the mitochondrial fusion. It is thought that the mitochondrial fusion is a basic mechanism on genetic recombination of mitochondrial genome. The processes of mitochondria and its nuclear fusions, reconstruction of the mitochondrial genome and segregation may be regarded as providing a kind of mitochondrial "meiotic" cycle.

There is no mitochondrial plasmid similar to mF. This suggests that the mitochondrial-fusion genes in other species are located on the cell nucleus. Moreover, no mutant for the mitochondrial fusion has been isolated in such species. The mF plasmid which is related with

mitochondrial fusion in *P. polycephalum* give great advantages for studying this phenomena. In the present study, only the gene for the plasmid's DNA polymerase has been elucidated. For other proteins coding on the mF plasmid, further work is needed to study their localizations and functions in mitochondria. It is not thinkable that only the genes on the mF plasmid cause the phenomena associated with the mitochondrial fusion. In addition to research on the genes of the mF plasmid, the genes which are related with mitochondrial fusion and located in the cell nucleus must be investigated.

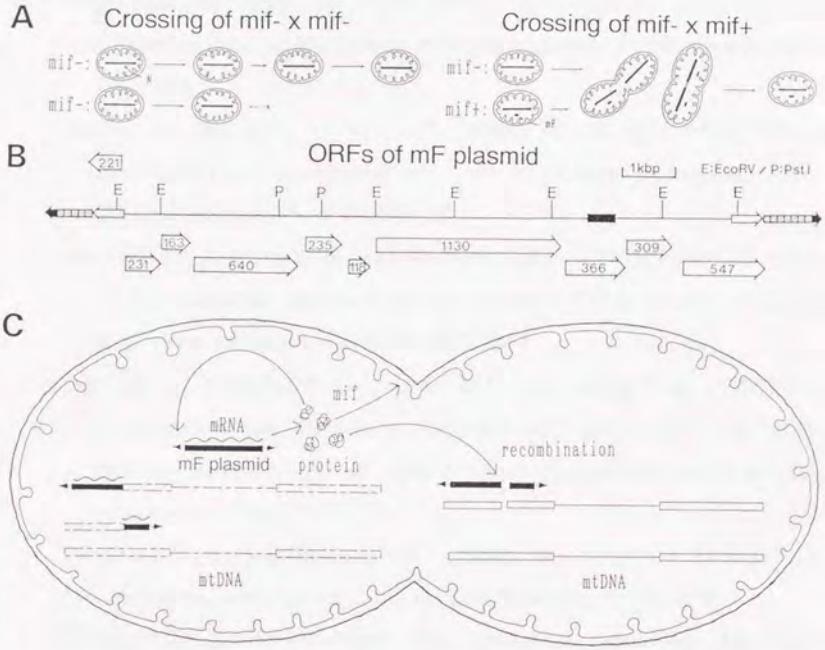

Fig. 28
A) In matings between the mif⁻ strains the mitochondrial fusion does not occur. On the other hand, in matings between the mif⁻ and mif⁺ strains, the mitochondrial fusion occurs. B) The mitochondrial fusion is caused by the mF plasmid. The mF plasmid has three TIRs and the identical sequence with the mtDNA and codes 10 ORFs. C) The mF plasmid is transcribed. The translated proteins of the mF plasmid may act on the mitochondrial fusion and recombination between the mtDNA and the mF plasmid.

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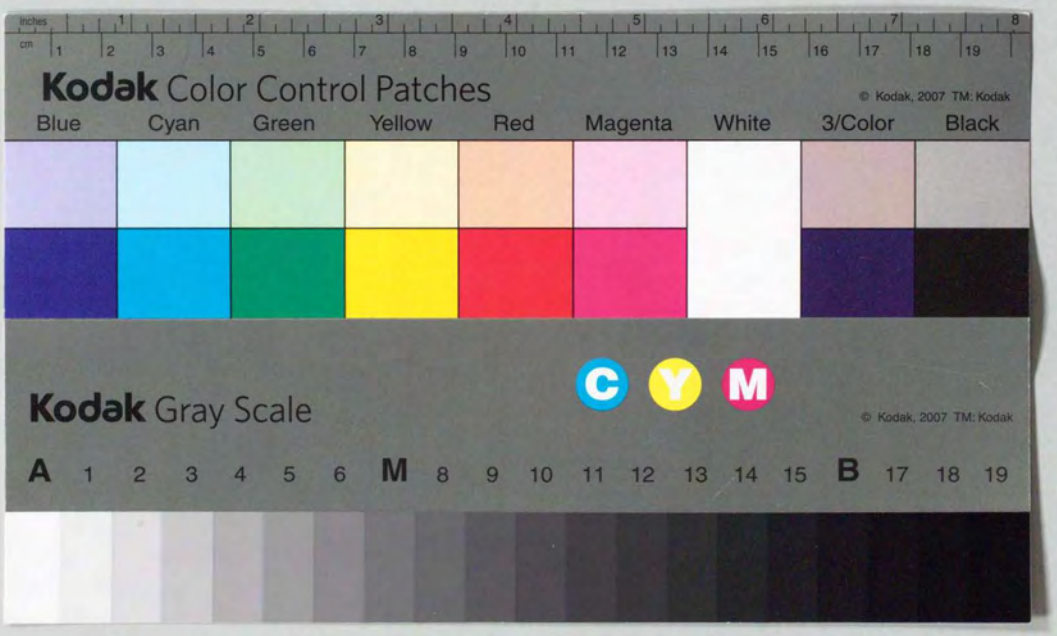
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