Sexuality of Mitochondria: Fusion, Recombination, and Plasmids

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Mitochondrial fusion, recombination, and mobile genetic elements, which are essential for mitochondrial sexuality, are well established in various organisms. The recombination of mitochondrial DNA (mtDNA) depends upon fusion between parental mitochondria, and between their mtDNA-containing areas (mt-nuclei), to allow pairing between the parental mtDNAs. Such mitochondrial fusion followed by recombination may be called "mitochondrial sex." We have identified a novel mitochondrial plasmid named mF. This plasmid is apparently responsible for promoting mitochondrial fusion and crosses over with mtDNA in successive sexual crosses with mF^- strains. Only in mF^+ strains carrying the mF plasmid did small spherical mitochondria fuse which subsequently underwent fusion between the mt-nuclei that contained the mtDNA derived from individual mitochondria. Several successive mitochondrial divisions followed, accompanied by mt-nuclear divisions. The resulting mitochondria contained recombinant mtDNA with the mF plasmid. Such features remind us also of the bacterial conjugative plasmids such as F plasmid. Therefore, in the final part of this chapter, we discuss the origin of sex and its relationship to the sexuality of mitochondria.

KEY WORDS: *Physarum polycephalum,* Mitochondria, Fusion, Recombination, Plasmid, Sex.

I. Introduction

Mitochondria are semiautonomous organelles with a large variety of functions in cellular metabolism. The question of how mitochondria are formed has fascinated us ever since Altmann (1890) first described these organelles 100 years ago. Initially, mitochondria were regarded as intracellular para-

sites. In the 1950s, when the resolving power of the electron microscope revealed the multitude of intracellular membranes in eukaryotes, mitochondria were frequently assumed to be just another intracellular membrane system composed of unit membranes. In the early 1960s, the discovery of mitochondrial DNA (mtDNA) led some investigators to propose that mitochondria were self-replicating units. Today, mitochondria are viewed as bona fide organelles that are very much controlled by the nucleus, yet they retain earmarks of their endosymbiotic past. They should harbor not only pieces of genes but also retain unique properties characteristic of ancient mitochondria and aerobic bacteria. One such property is a capacity for fusion and recombination.

The genetic properties of mitochondria have now been precisely described from the results of numerous crosses performed using various combinations of *ant*^{R} (resistance to antibiotics) mitochondrial markers of the yeast, *Saccharomyces cerevisiae.* Dujon (1981) concluded that mitochondrial crosses reveal all the properties of a multiple-copy genetic system, and can best be treated in terms of population genetics at the intracellular level. This model, although deliberately simplified in its formalism, was the first attempt to provide an integrated view of the genetic properties of mitochondrial crosses and gave rise to specific predictions amenable to quantitative experimental verification. One of its premises is random pairing and recombination between the different mtDNA molecules of a cell. Certainly, almost all data of mitochondrial crosses seem to indicate multiple rounds of pairing and recombination of mtDNA molecules as a panmictic pool. Such a situation suggests a cellular mechanism that promotes effective pairing and recombination of mtDNA. It may be simply explained as a result of mitochondrial fusion. Kuroiwa (1982) proposed that mitochondria perform "mitochondrial meiosis" during cell nuclear meiosis, since mitochondrial fusion and the following successive division are observed during meiosis of *S. cerevisiae.* However, we do not yet fully know how such mitochondrial fusion is regulated and by what kind of induction it occurs, though the past dozen years have witnessed the accumulation of a large body of knowledge on the biogenesis of mitochondria.

There are many reports (Table I), in which mitochondrial fusion is generally thought to be involved in pairing and recombination of mtDNA. In most reports, however, fusion and recombination of mtDNA have been reported independently, and practically no report exists which correlates these two phenomena, even in *S. cerevisiae,* whose genetic experimental systems have been well established. The simplest approach to demonstrating the relationship between these two phenomena, fusion and recombination, is to isolate a mutant that promotes or suppresses mitochondrial fusion. Recently, separate strains of the true slime mold *Physarum polycephalum,* one showing mitochondrial fusion and the other not, were isolated, and

these mitochondria were the first to show evidence of a relationship between fusion and recombination (Kawano *et al.,* 1991a). Furthermore, numerous other organisms display circumstantial evidence for mitochondrial fusion occurring with recombination of mtDNA. In this chapter, such mitochondrial fusion followed by recombination will be outlined first.

Mitochondrial fusion followed by recombination may be called "mitochondrial sex" or "mitochondrial meiosis" as proposed by Kuroiwa (1982). Historically, the term "sex" was used to describe the type of biparental reproduction exemplified by vertebrate animals. It is only relatively recently that the genetic consequences of sex have been understood along with an appreciation of its ubiquity among not only all eukaryote groups but also prokaryote groups. The term "mitochondrial sex" may also encounter some resistance, since all forms of "true" eukaryotic sex involve cycles of gamete fusion and meiosis with reproduction. However, an alternative view to sex and its origin now exists. Sex could have resulted from the evolution of parasitic DNA sequences that exploit opportunities for horizontal transmission, these opportunities being afforded by cycles of germ cell fusion and fission that allow horizontal spreading of sequences through populations (Hickey and Rose, 1988). Such parasitic elements could, in theory, enhance their own fitness by promoting gamete fusion. In other words, in an attempt to understand the origins of both the transfer of genes from one individual to another and the fusion of gametes, some researchers proposed that a parasitic gene might underlie the process.

Surprisingly, all strains of *P. polycephalum* that display mitochondrial fusion have a unique plasmid, a plasmid that promotes mitochondrial fusion (Kawano *et al.,* 1991a). This mitochondrial fusion-promoting (mF) plasmid is very similar to that proposed for the initial stage in the evolution of sex, as pointed out by Hurst (1991). The existence of an mF plasmid seems to provide direct support for the idea that parasitic, selfish genes are capable of manipulating their hosts (mitochondria) in the manner proposed by Hickey and Rose (1988). Numerous mitochondrial plasmids are well known in protists, fungi, and plants (Table II). They replicate themselves autonomously in the mitochondria independently of mtDNA and may be responsible for the diversity found in the mitochondrial genome. It is possible that such a scenario found in mitochondria reflects the initial stage in the evolution of parasitic sex.

The aim of this chapter is to summarize recent findings on the mitochondrial fusion mediated by the mF plasmid in *P. polycephalum.* The first part of the chapter will thus be concerned with general aspects of mitochondrial fusion and recombination. In the following section, the structure and function of the mF plasmid is presented together with observations of mitochondrial fusion during sporulation and plasmodium formation of *P. polycepha-*

lum. Finally, attention will be drawn to the origin of sex and its relationship to the sexuality of mitochondria.

II. A Morphological Aspect of Mitochondria

A. Giant Mitochondria

Mitochondria generally tend to be considered simply one of the small stationary structures found inside the cell, having an appearance like a drug capsule. This view may partly result from the influence of basic biology textbooks. Inside living cells, however, mitochondrial form is far from static, and harbored within these mobile and plastic organelles is an immense and varied repertoire of biochemical functions.

Since the end of the last century the existence of large filamentous structures in the cell, called "chondriomes," was well known from staining of tissue sections with iron hematoxylin. In the early part of this century, with the supravital demonstration of mitochondrial activity made possible using redox stains such as Janus green B and enzyme-catalyzed reduction of tetrazolium salts to colored and insoluble formazan compounds, some researchers successfully localized viable mitochondria in tissues. However, the insufficient resolving power of microscopes of that time and the granular cytoplasm of most unicellular organisms made the identification of single organelles difficult, leading to a dispute over whether these structures were simply artifacts or real organelles (Cowdry, 1918; Ernster and Schatz, 1981).

The first reliable information on the presence and behavior of mitochondria in living cells was derived from observations of well-spread cells in culture (chick fibroblasts) by Lewis and Lewis (1914). They were among the first to describe mitochondrial dislocation, shape changes, fission, and fusion. After the introduction of phase-contrast microscopy, many studies on mitochondrial morphology and motile activities appeared (Bereiter-Hahn, 1990; Bereiter-Hahn and Voth, 1994). These showed that mitochondrial form changes dynamically not only with the phases of development and differentiation or the rhythm of metabolism, but also with the physiological-chemical (osmotic pressure, ion concentration, etc.) environmental changes outside the cell and also with various experimental procedures. Phase-contrast images provide good lateral resolution and contrast. However, some nonmitochondrial cytoplasmic inclusions might give the same contrast, and in some cases difficulties arise in unambiguously identifying structures as mitochondria. Girbardt (1970) was the first to retrieve single mitochondria in fungal hyphae observed with the phase-contrast microscope and then identify them with the electron microscope.

In electron micrographs of thin sections, profiles of actively respiring mitochondria are rounded or elongated and are regularly distributed in the peripheral cytoplasm. The average cross-sectional diameter of the profiles is generally on the order of 0.3–0.4 μm. However, long filamentous forms, branching profiles, and dumbbell shapes are also typical of certain growth conditions or life-cycle stages (Stevens, 1981). Data such as the position of mitochondria in the cell or the arrangement of their cristae may be obtained from random thin sections. However, knowledge of the entire mitochondrial shape and population within a cell must be obtained by other means.

Since the latter half of the 1950s, a method of reconstructing electron microscopic images of a series of serial thin sections has been utilized for analyzing whole mitochondrial forms. The method is to prepare several consecutive thin sections that collectively cover the entire organelle and use the corresponding electron microscopic images to produce balsam or plastic plates, which can then be used to reconstruct the organelle and reveal its three-dimensional structures. Bang and Bang (1957) first used this method to analyze the mitochondria in human hepatocytes with complex ramification. Osafune (1973) analyzed three-dimensional structures of mitochondria and other cell components based on about 50 serial thin sections of synchronously grown *Euglena gracilis* cells, and found that giant mitochondria with markedly irregular shapes and multiple branching formed during the growth phase of the cell cycle. The matrix of giant mitochondria was very large in volume and no cristae extended into the middle of the matrix. These authors suggested that a giant mitochondrion is formed by fusion of smaller mitochondria, that its newly formed matrix is further enlarged, and that it may continually change its configuration. Spurred by such studies, many investigators analyzed the morphological changes of mitochondria by this serial thin section method and observed that giant mitochondria spread inside the cells of many organisms. Particularly in protists, algae, and fungi, a variety of giant mitochondrial forms have been reported (Table I).

B. Mitochondrial Fusion

A ramified giant mitochondrion resembles the resultant product of mitochondrial fusion. However, when a cell has giant mitochondria, it cannot immediately be assumed that the giant mitochondrion is the product of mitochondrial fusion because, if a cell always has giant mitochondria regardless of cell cycle, life cycle, or growth conditions, the giant mitochondria cannot be the result of fusion. If the giant mitochondria result from fusion, there must be a continuously changing pattern from small to giant mitochondria, and their appearance and disappearance must show a certain periodic-

ity. In some of the reports on giant mitochondria, this periodicity has not been confirmed, so it is not true that all reports about giant mitochondria suggest mitochondrial fusion (Table I).

The study of structural organization of mitochondria in a yeast, *Saccharomyces cerevisiae,* offers a unique opportunity to relate the considerable metabolic, genetic, and biochemical data on this organism to visible morphological entities (Pon and Schatz, 1991). A series of morphological studies on mitochondrial behavior in *S. cerevisiae* were reported first by Yotsuyanagi (1962a,b). He studied the mitochondrial morphology of each phase of the yeast by staining with Janus green, and reported that giant mitochondria occur at the transitional phase between the exponential growth phase and the stationary growth phase. The changes in cellular respiration are paralleled by distinctive changes in mitochondrial morphology.

The presence of a single, highly branched, giant mitochondrion in each cell was first proposed by Hoffman and Avers (1973). Subsequent studies have demonstrated a more complex situation. In general, it has been shown that the giant mitochondrion is not a static entity, but that its form and volume undergo rapid and extensive modifications in accord with changes in the life cycle and physiological state. Examination and measurement of mitochondrial profiles in serial thin sections of 34 whole cells, from several different strains growing vegetatively under a variety of conditions, led to the following basic conclusions (Stevens, 1977): The three-dimensional form varies, with small oval shapes predominant in stationary-phase cells, and some elongated, curved, and branching forms in all states. However, one mitochondrion, or in some rare cases two, is always significantly larger and more complex than the others in the cell. The volume of the giant mitochondrion is clearly a function of the physiological state of the cell: In glucose-repressed cells, it represents only about 3% of the cell volume, whereas in fully respiring cells, it represents 10–12%. These values are in agreement with those given by Grimes *et al.* (1974) and Damsky (1976). In these observations and those on sporulating cells, it appears that the giant mitochondrion exists in a mobile and flexible state within the living yeast cell. It forms a branching network that ramifies throughout the peripheral cytoplasm; its branches constantly fuse together, pinch apart, and change location. The membranes, enzymes, and nucleic acid components are thus being continually redistributed within a "fluid" mitochondrial system.

The most powerful tools for detecting and identifying the periodicity of mitochondrial fusion *in situ* are advanced fluorescence techniques. Fluorochroming endows the organelles with the luminescence. Thus, very fine extensions below the resolving power of a light microscope can be detected because of their fluorescence. In addition, spatial or temporal variations of the fluorescence emission along a single mitochondrion indicate changes

TABLE I

Organisms in Which Giant Mitochondria, Mitochondrial Fusion, Mitochondrial-Nuclear Fusion, and Recombination of mtDNA Are Reported

Organisms	Mitochondria		Mt-nuclei fusion	Recombination	Reference
	Giant	Fusion			
Protists					
Blastocrithidia culicis	+				1
Cryptobia vaginalis	+				2
Trypanosoma brucei	+				3
Trypanosoma cruzi	+				1
Fungi					
Blastocladiella emersonii	+	+			4
Bullera alba	+	+			5
Candida albicans	+	+			6
Candida utilis	+				4, 7
Olpidium brassicae	+				8
Pityrosporum orbiculare	+				9
Physarum polycephalum	+	+	+	+	10–12
Saccharomyces cerevisiae	+	+	+	+	13–18
Schizosaccharomyces pombe	+				19
Algae					
Chlamydomonas reinhardtii	+	+		+	20–25
Chlorella fusca	+				26
Chlorella minutissima	+				27
Chlorococcum infusionum	+	+			28
Chromuliona pusilla	+				29
Eudorina illinoiensis	+				30
Euglena gracilis	+	+			31–38
Friedmannia israelensis	+				39
Hemiselmis rufescens	+				40
Hydrodictyon reticulatum	+	+			41
Pleurochrysis carterae	+				42
Polytoma papillatum	+	+			43–45
Polytomella agilis	+	+			46
Prorocentrum minimum	+				47
Pyramimonas gelidicola	+				48
Rhodella reticulata	+				49
Higher plants					
Pelargonium zonale	+	+	+		50, 51
Zea mays	+				52
Animals					
Xenopus endotherial cells	+	+			53
Rat liver cells	+	+			54
Rat heart cells	+	+			55
Mouse cultured cells (3T6)	+	+			55
Mouse lymphocytes	+	+			56
Mouse ascites tumor cells	+	+			57
Human alcoholic liver cells	+	+			58

(*continues*)

TABLE I (*continued*)

Note: It is well known that mitochondrial fusion occurs during spermatogenesis of mosses, ferns, and animals but such events are omitted from this table. It is also well known that the recombination of mtDNA occurs in the hybrid cells of plants but such events are omitted from this table.

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within the inner compartment. Most of the fluorochromes used for *in situ* staining of mitochondria are lipophilic cationic dyes. Alkylated nitrogen seems to add to the organelle specificity of dyes. The most widely used group of dyes is the rhodamines, first used by Johannes (1941) to stain mitochondria in plant cells. Improvement of purification methods allowed more detailed studies on the properties of different rhodamines, among which the laser dye rhodamine 123 proved to be least toxic and with the highest specificity for staining mitochondria (Johnson *et al.*, 1980). Other groups with similar properties are the dimethylaminostyryl-pyridinium-iodide derivatives with methyl and ethyl groups (DASPMI and DASPEI) and cyanine dyes (DiOC6, 3,3′-dihexyloxocarbocyanine iodide; DiOC7, 3,3′-dihectaloxocarbocyanine iodide). They have been found to accumulate in mitochondria, making them useful for studying mitochondria *in situ* and isolated in suspension (Chen, 1988, 1989).

On the basis of such improved methods, systematic changes in mitochondrial morphology during the cell cycle and life cycle have been reported for many organisms. In general these changes follow a common series of events: at the onset of interphase the giant mitochondrion consists primarily of one highly reticulated basket-shaped complex that lines the periphery of the cell. During interphase the size of the mitochondrial basket increases, as does the number of additional small mitochondria. During mitosis, the mitochondrial basket is subdivided into several fragments that tend to form clusters, and after cytokinesis the total number of mitochondria is drastically reduced again. Organisms exhibiting such a cycle include *Saccharomyces, Candida, Schizosaccharomyces, Euglena, Chlamydomonas, Chlorella, Chlorococcum, Hydrodiction,* and the dinoflagellate *Prorocentrum minimum* (Table I).

The alternation of mitochondrial networks with fragmentation to individual mitochondria can change the distribution of metabolites and thus create a specific environment for the nucleus. However, this alternation does not seem to be essential for progression through the cycle, because Hayashi and Ueda (1989) found no indication for such a cycle in the mitochondrial organization of *Euglena,* which had previously been reported to exist in this genus. On the other hand, in cultured animal cells, fusion of mitochondria can easily be observed with phase-contrast and fluorescence microscopy. Johnson *et al.*, (1980) demonstrated an interconnecting mitochondrial network in a mouse 3T6 cell using rhodamine 123. Bereiter-Hahn (1983) analyzed fusion of two mitochondria in an amphibian endothelial cell in culture by time-lapse filming. They found that fusion may occur either when two mitochondria collide with their tips and then fuse, or one mitochondrion touches the side of another with its tip, resulting in fusion. The mitochondrial fusion events observed with cultured animal cells seem not to depend

on a certain physiological condition; rather, the frequency of such events is related to mitochondrial motility, which does not show cell cycle periodicity.

C. Mitochondrial Nuclear Fusion

There are two types of mitochondrial fusion: One is periodical, depending on the cell cycle and/or the life cycle as observed in protists, algae, and fungi; while the other is accidental, as observed in cultured animal cells. Of course, in the later case, mitochondria may fuse after several efforts, i.e., the tip of one mitochondrion may slide along the side of another or the two may approach each other several times (Johnson *et al.*, 1980; Bereiter-Hahn and Voth, 1994). Electron micrographs show mitochondria which approached each other very closely and behaved as if they were about to fuse though such an event can never be foreseen in fixed cells. The membranes in the contact zone exhibit a high electron density. The connection starts at contact sites between the inner and outer membrane, which are highly specialized zones where hexokinase and creatine kinase are localized (Biermans *et al.*, 1990; Nicolay *et al.*, 1990; Brdiczka, 1991). The structure of contact zones may predispose them for a fusion event, as suggested by Bereiter-Hahn and Voth (1994).

It is important to understand with respect to the genetic requirements whether the mitochondrial fusion in either case is accompanied by redistribution of the mitochondrial nuclei (mt-nuclei; synonymous with mitochondrial nucleoid; Kuroiwa, 1982; Kuroiwa *et al.*, 1994), since this would provide strong morphological support for the considerable genetic data concerning recombination and segregation of mitochondrial genomes in crosses. From serial thin sections, however, it has not been shown that mt-nuclei form a continuous reticulum inside a giant mitochondrion, even in *S. cerevisiae.* This may be because visualization of mtDNA *in situ* in thin sections is greatly dependent on the fixation employed and the physiological state of the organism, and is very difficult to observe throughout the mitochondrial fusion event (Stevens, 1981). On the other hand, the introduction of a DNA-specific fluorochrome such as 4′-6-diamidino-2-phenylindole (DAPI) in biological research opened new horizons in the dynamics of mt-nuclei. DAPI was originally used as a highly sensitive and specific fluorescent probe for mtDNA in *S. cerevisiae* (Williamson and Fennell, 1975) and chloroplast DNA in plants (James and Jope, 1978; Coleman, 1978). The blue-white fluorescence of DAPI is stronger than that obtained with other dyes.

In *S. cerevisiae,* with the DAPI staining technique, mitochondrial nuclei have been observed as small, discrete, fluorescent spots. They have been observed to appear as a "string of beads" during periods of exponential

growth (Williamson and Fennell, 1975, 1979). With the fluorescent dyes DAPI and DASPMI, Sando *et al.*, (1981) and Miyakawa *et al.* (1984) demonstrated that the morphology of yeast mitochondria changes even more dramatically during meiosis and sporulation. In zygotes just after mating, 50–70 mt-nuclei are separated from each other, and each spherical mitochondrion contains only one mt-nucleus. In the later stage of premeiotic DNA synthesis, a single branched giant mitochondrion is formed as a result of complete mitochondrial fusion. All of the mt-nuclei are arranged in an array on a giant mitochondrion and coalesced into a string-like network. Through meiosis I and II, strings of mt-nuclei are observed close to the dividing nuclei. At late meiosis II, a ring of mt-nuclei enclosing each daughter nucleus is formed. In ascospores, discrete small mt-nuclei appear close to each spore as a string of beads. Many mt-nuclei are excluded from the ascospores and remain in the residual cytoplasm of the ascus. Recently, Miyakawa *et al.*, (1994) developed a simple and rapid method for double vital staining of mitochondria and mt-nuclei with DiOC6 and DAPI, and confirmed the relationship between mitochondrial fusion and mt-nuclear fusion in living yeast cells.

The mt-nuclear fusion following mitochondrial fusion or within a giant mitochondrion has been confirmed only in *S. cerevisiae* and *P. polycephalum* (Section IV). In spite of many reports of mitochondrial fusion events and giant mitochondria (Table I), there are no reports that the mt-nuclei fuse in other organisms as drastically as in *S. cerevisiae.* Most of the giant mitochondria reported to date in a variety of organisms were confirmed by serial thin sections. It is almost impossible with electron microscopy to analyze precisely the location, shape, and extent of mt-nuclei and to demonstrate the mt-nuclear fusion event as described by Stevens (1981) and Kuroiwa (1982). This may be one of the reasons for the lack of evidence of mt-nuclei fusion. Moreover, if mitochondria have only a small amount of mtDNA, even by the DAPI staining technique, it is not easy to monitor the behavior of mt-nuclei during mitochondrial fusion events. This also applies to cultured human cells (Satoh and Kuroiwa, 1993). It is more important to understand whether mitochondrial fusion events are accompanied by mt-nuclear fusion than whether they are periodic or accidental events. If it is not accompanied by mt-nuclear fusion, mitochondrial fusion is no better than an accident with respect to genetic requirements.

Studies on mitochondrial fusion have been limited to unicellular or simple organisms such as protists, algae, fungi, and animal cells, including even cultured cells. It is very difficult with conventional microscopy to monitor mitochondrial fusion that accompanies mt-nuclear fusion in cells within tissues and organs of animals and plants. Recently, Kuroiwa *et al.*, (1990, 1991a) developed a new method that enables observation of organelle nuclei in sections prepared in a resin (Technovit 7100) permeable to solutions of

DAPI. This method allows observation of an extremely small amount of organelle DNA (less than 100 kbp) in the cells within tissues and organs, and also enables observation of giant mt-nuclei in the young ovules of *Pelargonium zonale.* The giant mt-nuclei around a nucleus appear in some areas of the young integument. Since the number of giant mt-nuclei per cell is fairly low, it is possible that they are formed not only by preferential mtDNA synthesis but also by the association of small, discrete mt-nuclei. Transformed and giant mitochondria have been observed in the egg cells of some plants: *Zea mays* (Diboll and Larson, 1966), *Pteridium aquilinum* (Tourte, 1975), and *Crepis capillaris* (Kuroiwa, 1982). The three-dimensional reconstruction of isolated egg cell protoplasts of *Z. mays* showed that the giant mitochondria are organized in filamentous and reticulate structures located near the nucleus in the center of the cell (Faure *et al.,* 1992). Kuroiwa and Kuroiwa (1992) demonstrated that such giant mitochondria contain giant mt-nuclei which contain large amounts of DNA (about 4 Mbp) in the Technovit sections of the egg cells of *P. zonale.* Although it is not yet known whether the giant mt-nuclei arose from mt-nuclear fusion or amplification of mtDNA during megagametogenesis, their presence is significant and may enable analysis of the mechanism of recombination and rearrangement of the plant mitochondrial genome as reported elsewhere (Lonsdale *et al.,* 1988; Lonsdale, 1989; Schuster and Brennicke, 1994).

III. A Genetic Aspect of Mitochondria

A. Mitochondrial Genetics

Much has been learned about the rules and mechanisms that govern inheritance of mitochondrial and chloroplast genomes. In many organisms, these organelle genomes are transmitted to progeny predominantly or entirely by only a single parent. This uniparental mode of inheritance has usually been attributed in oogamous species to failure of organelles from the male gamete to enter the egg, or to the presence in the male gamete of comparatively few organelles. Thus, the determination of organelle transmission in such species is dependent upon sexual differentiation. In isogamous species, such as *Saccharomyces cerevisiae,* uniparental inheritance of mitochondrial markers is thought to be due, at least in part, to vegetative segregation; for example, to random partitioning of mitochondria by cell division during sexual development (Birky, 1978; Birky *et al.,* 1982).

Mitochondrial genetics is a major category of transmission genetics. Mitochondrial genes differ at the molecular level from eukaryotic cell nuclear

genes in a number of interesting aspects of structure and function. Even more striking are the differences in patterns of inheritance: cell nuclear genes show biparental inheritance while mitochondrial genes most often show uniparental inheritance; cell nuclear alleles segregate only during meiosis with rare exceptions while mitochondrial alleles segregate with high frequency at mitosis as well as meiosis. This phenomenon is called vegetative segregation.

Genes in mitochondria and chloroplasts were first identified by the two unique features that distinguished them from cell nuclear genes: uniparental inheritance and vegetative segregation. A goal of mitochondrial genetics is to explain these two phenomena at the molecular level, in terms of the behavior of mitochondria and mtDNA molecules.

The transmission of mitochondria in isogamous species such as *S. cerevisiae* is most fruitfully dealt with as a problem in population genetics at the intracellular level. Among the parameters and phenomena that must be considered are the input frequencies of alleles in the populations; the mating structure of the populations, e.g., the degree of "panmixis"; random drift of gene frequencies; and the extent of migration of DNA molecules between populations. For reviews of such transmission genetics of mitochondria and chloroplasts, see Sager (1972), Gillham (1978), Dujon (1981), Wilkie (1983), and Birky (1983a,b, 1991). In mitochondrial inheritance, however, additional peculiarities and complexities exist, mainly resulting from some mobile genetic elements and mitochondrial fusion. The situation is thus somewhat complex. In this chapter, primarily recombination and other such mobile genetic elements of mitochondria are reviewed.

B. Recombination

Mitochondria are part of the cell, not distinct organisms, and one cannot score the phenotype of mitochondria but only the phenotype of the cell in which they reside. The first mitochondrial point mutations to be isolated were those of antibiotic resistance (*ant*^{R} mutation) in *Saccharomyces cerevisiae* (Linnane *et al.*, 1968; Thomas and Wilkie, 1968), and it was immediately apparent that determination of frequencies of recombination between these mutations would not alone permit construction of a complete genetic map, since rates of recombination can reach a maximum of 20–25% within only 6000 bp or less (Dujon, 1981). Mitochondrial genomes of *S. cerevisiae* are capable of recombination and sorting out. When haploid yeast strains of opposite mating type fuse to form zygotes, mtDNAs from each parent mix and many undergo one or more rounds of pairing. After about 20 cell generations, recombinant and parental genomes are fully segregated among the diploid progeny so that all of the 100 or so mtDNA molecules in any

given cell are identical. All cells are therefore homoplasmic with respect to their mitochondrial genomes.

These observations led to the following model of yeast mitochondrial genetics—an analogy between yeast mitochondrial and phage crosses (Dujon, 1981). This model assumes that a fully mixed (panmictic) pool of mtDNA is established rapidly in zygotes and that mtDNA molecules in the zygote undergo repeated rounds of random pairing and recombination. At cell division, the mtDNA molecules are partitioned between mother and bud with the same degree of randomness, so that eventually each cell is homoplasmic for molecules of one genotype or another. Although recombination events continue in the diploid cells, when these cells are homoplasmic or nearly so, all matings will occur between genetically identical molecules and no additional detectable recombinants will be produced. Cell division and vegetative segregation thus limit the number of rounds of effective mating and recombination. Furthermore, all genotypes of molecules replicate at approximately the same rate in all cells. Consequently, the output frequency of a particular allele is equal to the input frequency. Based as it was on random diploid analysis, this model made no provision for differences in the behavior of individual zygotes.

Strausberg and Perlman (1978) investigated segregation of mitochondrial genomes in zygotes by partial pedigree analysis of crosses of *S. cerevisiae.* Clones derived from first end buds are usually pure (or nearly so) for a parental genotype, reducing the occurrence of detectable recombination of mitochondrial markers in these zygotes. Cells derived from a zygote after removal of the first end bud are predominantly of the other parental genotype. The position of the first bud is important in determining the transmission of mtDNA and the frequency of recombination. Aufderheide and Johnson (1976) have studied the movement of mitochondria from zygotes to buds by phase-contrast microscopy and found little mixing of the zygote cytoplasm before formation of the first bud. This is one reason why the initial end buds are pure for the mitochondrial parental type contributed by the haploid cell which formed that end of the zygote (Wilkie, 1983).

However, zygotes with central buds appearing first may most closely resemble the panmictic situation as Dujon *et al.* have envisioned it (Dujon, 1981). A first central bud receives cytoplasm from a part of the zygote in which both types of parental mtDNA molecules should be present and also usually yields some recombinant progeny. After formation of the first bud, the cytoplasm apparently mixes more fully so that a panmictic pool is more closely approximated at that time, even for subsequent end buds. Although the behavior of individual mitochondria during zygote formation and budding has not been well studied, the recombination of mtDNA should depend upon fusion between parental mitochondria and between their mt-nuclei

to allow pairing between the parental mtDNAs (Sando *et al.,* 1981; Miyakawa *et al.,* 1984; Tanaka *et al.,* 1985).

C. Mobile Genetic Elements

1. Mobile Intron

Recombination among most loci on the mitochondrial genome of *S. cerevisiae* appears reciprocal at the population level, with parental markers transmitted coordinately to the diploid progeny as described earlier. However, a particular phenomenon, called "polarity of recombination," occurs in a specific region (the polar region) of the mitochondrial genome in the immediate vicinity of the locus ω. Since the first ant^R mutants (cap^R and ery^R) found localize in this region, this phenomenon has played an important role in elucidating the mechanisms involved in mitochondrial crosses (Bolotin *et al.,* 1971). We now know that the polar region contains the gene for the large rRNA with cap^R and ery^R being point mutations in this gene, which codes for a mobile group I intron encoding endonuclease (Lambowitz, 1989; Scazzocchio, 1989; Dujon, 1989; Lambowitz and Belfort, 1993).

Group I introns form a structural and functional group of introns with widespread but irregular distribution among very diverse organisms and genetic systems. Evidence has now accumulated that several group I introns are mobile genetic elements with properties similar to those originally described for the ω system of *S. cerevisiae:* mobile group I introns encode sequence-specific, double-stranded endonuclease, which recognizes and cleaves intronless genes to insert a copy of the intron by a double-stranded break repair mechanism. Group I introns are of special interest for two reasons. The first reason, shared with group II introns, is that several group I introns are capable of self-splicing *in vitro* (Cech, 1988). The second interesting aspect of group I introns, and so far specific for this group, is their ability to propagate themselves by self-insertion at predetermined positions into intronless sites of the genes. This phenomenon, which originally was discovered with the intron of the mitochondrial large subunit rRNA (LSU) gene of *S. cerevisiae,* has recently been shown to occur with several other group I members. These include mitochondrial, chloroplast, nuclear, and prokaryotic introns of very diverse organisms such as, in addition to yeast, *Chlamydomonas, Neurospora, Physarum, Coprinus,* and bacteriophage T4 (Dujon *et al.,* 1989; Lambowitz and Belfort, 1993).

Genetic crosses between ω^+ and ω^- strains also revealed that the ω^+ allele itself is transmitted to more than 95% of the progeny, the ω^- allele being almost completely eliminated. At this time, a double-stranded break forms within the intronless LSU gene at a location corresponding to the intron

insertion site (Jacquier and Dujon, 1985; Macreadie *et al.*, 1985). This break disappears after a few hours when zygotes age and bud, allowing different mtDNA molecules to segregate out. Zinn and Butow (1985) have determined the kinetics of ω recombination. Within 2–4 hr after ω^+ and ω^- are mixed, a nonparental form of the LSU gene appears that contains the 1.1 kbp intron but not the region flanking the ω^+ parental allele. Although this recombinant is not a major product in the conversion of ω^- to ω^+, it serves as a convenient measure of ω recombination. Since zygotes do not appear until about 2 hr after cells of opposite mating type are mixed, mitochondria from each parent must therefore fuse and the parental mtDNAs become readily available for recombination essentially as soon as zygotes are formed. This conclusion does not conflict with the fluorescent-microscopic observation that mt-nuclei accumulate on a thread-like mitochondrion and disperse into many small ones just after mating (Sando *et al.*, 1981; Miyakawa *et al.*, 1984). If the mt-nucleoids are fully mixed in the zygote cytoplasm, the random arrangement of mt-nucleoids from both parents on a giant mitochondrion would allow significant freedom of access between mtDNAs and extensive recombination of neighboring mtDNAs from ω^- and ω^+.

2. Mitochondrial Plasmid

The size and structural complexity of fungal mitochondrial genomes occupy a middle ground between those of metazoa and higher plants. The organization of genes is not as frugal as that in animal mtDNA nor as loose as that in plant mitochondrial genomes. Although mtDNAs from some fungi match those from vertebrates in the number of encoded genes, this is not so for *S. cerevisiae* (Clark-Walker, 1992). However, the relative poverty of fungal mitochondrial genes is redressed in some species by a richness in structural complexity of intergenic regions and also by the presence of optional introns such as ω. Some fungal mitochondria also carry, in addition to a high-molecular-weight DNA component that represents the main mtDNA, smaller circular and linear DNA molecules such as mitochondrial plasmids. 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 (Samac and Leong, 1989; Lonsdale, 1989; Meinhardt *et al.*, 1990; Griffiths, 1992).

Linear plasmids in eukaryotes have been identified in numerous fungi, protozoa, and plants, and most of them appear to be localized in the mitochondria. 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.,* 1990). The almost complete nucleotide sequences of some linear mitochondrial plasmids have been determined, such as S1 and S2 in maize, pClK1 in *Claviceps purpurea,* pMC3-2 in *Morchella conica, kalilo* in *Neurospora intermedia, maranhar* in *N. crassa,* and pAL2-1 in *Podospora anserina* (Table II). They encode DNA and/or RNA polymerase and a few short open reading frames (ORFs) as follows: DNA polymerase and two short ORFs on S1, RNA polymerase and a short ORF on S2, DNA and RNA polymerases and four short ORFs on pClK1, DNA polymerase and a short ORF on pMC3-2, DNA and RNA polymerases on *kalilo,* DNA and RNA polymerases and three short ORFs on *maranhar,* and DNA and RNA polymerases on pAL2-1.

Circular plasmids in eukaryotes have been also identified in the mitochondria of numerous fungi and plants (Esser *et al.,* 1986; Lonsdale *et al.,* 1988). Extensive work on circular plasmids has been carried out, particularly using *Neurospora.* Based on DNA hybridization studies, most of the circular mitochondrial plasmids of *Neurospora* have been placed in one of three homology groups that are named after the geographical location where the initial isolate was found (Collins *et al.,* 1981; Stohl *et al.,* 1982; Natvig *et al.,* 1984). The circular plasmids studied thus far encode plasmid-specific polymerases (Li and Nargang, 1993). Plasmids of the Mauriceville homology group encode a reverse transcriptase that appears to be involved in replication of the plasmid (Nargang *et al.,* 1984; Kuiper and Lambowitz, 1988). The lone plasmid from the LaBelle homology group contains an open reading frame in which motifs characteristic of reverse transcriptase have been identified, though the lack of some highly conserved residues in the motifs is noted (Pande *et al.,* 1989). The subsequent discovery that the LaBelle plasmid encodes a DNA-dependent DNA polymerase prompted a search for motifs characteristic of DNA-dependent DNA polymerases (Schulte and Lambowitz, 1991). Some similarity to the DNA polymerase family B has been found, but the reverse transcriptase motifs are considered more convincing. ORFs encoding the DNA polymerase family B are also found in some other linear mitochondrial plasmids.

Although extensive studies on sequencing of some mitochondrial plasmids has been performed, the reason for the existence of plasmids is still something of a mystery (Yang and Griffiths, 1993). Most plasmids appear

TABLE II
Mitochondrial Plasmids

Organisms	Name	Size (kbp)	Structure	TIR (bp)	TP	Gene	Reference
Protists							
Paramecium caudatum	Types I, II, III	8.9-1.4	Linear	Yes	Yes		1, 2
P. jenningsi	Type I	6.0, 6.8					2
P. multimicronucleatum	Type I	6.0, 6.8					2
P. polycaryum	Type II	8.2-1.4					2
Fungi							
Agaricus bitorquis	pEM	7.4	Linear	1000	—	D, R-pol	3, 4
	pMPj	3.7	Linear	—	—		3
Ascobolus immersus	pAI2	5.6	Linear	700	Yes	D-pol	5, 6
	pAI3	2.8	—				5
Ascochyta rabiei	—	13.0	Linear	—	—		7
Claviceps purpurea	pClB4	6.7	Linear	—	Yes		8
	pClK1	6.8	Linear	327	Yes	D, R-pol	9
	pClK2	5.5	Linear	—	No		8
	pClK3	1.1	Linear	—	No		8
	pClK9	6.7	Linear	—	Yes		8
	pClT5	7.1	Linear	—	Yes		8
Fusarium merismoides	—	2.1	—				10
	—	1.8	—				10
Fusarium oxysporum f. sp. *conglutinans*	pFOXC2	1.9	Linear	—	Yes		11
Fusarium solani	pFSC1	9.2	Linear	—	—		12
f. sp. *cucurbitae*	pFSC2	8.3	Linear	—	—		12
Fusarium sporotrichoides	—	2.1	Linear	—	—		13
Gaeumannomyces graminis	E1	8.4	Linear	—	—		14
	E2	7.2	Linear	—	—		14
Lentinus edodes	pLLE1	11.0	Linear	—	—		15
Morchella conica	—	6.0	Linear	750	—		16, 17
Neurospora crassa	*maranhar*	7.0	Linear	—	Yes		18, 19
	Mauriceville	3.6	Circular			RT	20–22
	Roanoke	5.2	Circular				23
Neurospora intermedia	*kalilo*	8.6	Linear	1366	Yes	D, R-pol	24, 25
	Fuji	5.2	Circular				23
	LaBelle	4.1	Circular			D-pol	26
	Varkud	3.8	Circular			RT	27
Neurospora tetrasperma	Hanalei	5.2	Circular				23
Pleurotus ostreatus	pLPO1	10.0	Linear	—	Yes		28
	pLPO2	9.4	Linear	—	Yes		28
Physarum polycephalum	mF	16.0	Linear	144	Yes	D-pol	29, 30
Podospora anserina	pAL2-1		Linear				31
Tilletia caries	pTCC	7.4	—				32
Tilletia controversa	pTCT	7.4	Linear	—	Yes		32
Tilletia laevis	pTCL	7.4	—				32
Higher plants							
Beta vulgaris	Mc.a	1.6	Circular				33
	Mc.d	1.3	Circular				33
	pO	1.4	Circular				34
	—	7.3	Circular				35
	—	1.4	Circular				35

(*continues*)

TABLE II (*continues*)

Organisms	Name	Size (kbp)	Structure	TIR (bp)	TP	Gene	Reference
Brassica compestris	—	11.5	Linear	325	Yes		36
Helianthus annuus	P1	1.4	Circular				37
	P2	1.8	—				37
	P3	1.8	—				37
Oryza sativa	B1	2.1	Circular				38
	B2	1.5	Circular				38
	B3	1.5	Circular				39
	B4	1.0	Circular				40
	L1	1.0	Circular				41
	L2	1.4	Circular				41
	L3	1.4	Circular				41
	L4	2.1	Circular				41
Sorghum bicolor	N1	5.8	Linear	—	Yes		42
	N2	5.4	Linear	—	Yes		42
	—	2.3	Circular				43
	—	1.7	Circular				43
	—	1.4	Circular				43
Vicia faba	—	1.7	Circular				44
	—	1.7	Circular				44
	—	1.5	Circular				44
Zea diploperennis	D1	7.4	Linear	—	Yes		45
	D2	5.4	Linear	—	Yes		45
Zea mays S-cytoplasm	S1	6.4	Linear	208	Yes	DNA pol	46, 47
S-cytoplasm	S2	5.5	Linear	208	Yes	RNA pol	48, 49
RU-cytoplasm	R1	7.5	Linear	187	Yes		50
RU-cytoplasm	R2	5.4	Linear	187	Yes		50
N,S,C-cytoplasm	—	2.3	Linear	170	—	tRNAs	51
N,S,C-cytoplasm	—	1.9	Circular				52
C-cytoplasm	—	1.6	Circular				53
C-cytoplasm	—	1.4	Circular				54

Note: D-pol, DNA polymerase; R-pol, RNA polymerase; RT, reverse transcriptase; —, not decided.

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to be neutral passengers with no obvious detrimental effect on their hosts. In such a sense, they are selfish. Two linear plasmids are unique in that they cause the death of their hosts by inserting into mitochondrial DNA, which disrupts mitochondrial function; these are referred to as senescence plasmids (Griffiths, 1992). Under conditions of prolonged growth at 37°C, the circular plasmids Mauriceville and Varkud occasionally insert themselves into mtDNA, resulting in mitochondrial malfunction and growth irregularities (Akins *et al.*, 1986), but these and other circular plasmids do not confer the strong predisposition to death shown by the linear *kal*DNA and *mar*DNA plasmids. For example, it is known that the senescence in *N. intermedia* and *N. crassa* is induced by the integrations of *kalilo* and *maranhar* plasmids into the mtDNAs, respectively. However, these plasmids encode only DNA and RNA polymerases, and do not encode any genes which cause senescence.

Certainly one of the predominant impressions that has emerged from the present work is that plasmids are the rule in *Neurospora,* and not the exception. The types that we have identified are undoubtedly merely the tip of an iceberg of diversity. The liner plasmids so far described in fungi and plants, despite showing no nucleotide homology, have remarkably similar general structures, which apparently code for the same kinds of

proteins related to viral polymerases, and are virtually all mitochondrial in location. Assuming that all linear plasmids will have such properties, is it reasonable to propose that this entire gamut of plasmid types all evolved from one common ancestor that inhabited the original endosymbiont that gave rise to mitochondria? Alternatively, it is possible that only one structure compatible with the selfish DNA lifestyle exists within mitochondria, and that the multitude of types have converged on this form. Thus, linear plasmids are most probably descendent from viral genomes. The demonstration of the existence of virus-like capsids could prove this supposition, as proposed by Meinhardt *et al.* (1990). It is reasonable that bacteriophages are progenitors of the linear plasmids of bacteria. The same may be true for the mitochondrial linear plasmids since it is generally accepted that mitochondria are remnants of endosymbiotic bacteria.

3. Mobile Genetic Elements and Mitochondrial Fusion

Uniparental inheritance of mtDNA is virtually axiomatic in animals, plants, and fungi. Generally, during sexual reproduction in these organisms, only the female parent transmits mitochondria to its progeny, and the maternal lineage of mtDNA is strictly maintained. In contrast, mobile introns, such as ω of *S. cerevisiae* (Zinn and Butow, 1985; Dujon, 1989) and the ω-like intron of *C. smithii* (Boynton *et al.*, 1987; Remacle *et al.*, 1990), promote their insertion into the mtDNA, and as a result, spread through the mitochondrial population. Moreover, many plasmids are known in fungal mitochondria, and some of them may contain ω-like introns (Nargang *et al.*, 1984). Mobile genetic elements such as ω-like introns and plasmids seem to have a scattered distribution within and among species against a background of uniparental mtDNA lineage (Dujon, 1989; May and Taylor, 1989; Collins and Saville, 1990). In particular, ω-like introns are sporadically distributed, not only in mitochondria, but also in chloroplasts, in nuclear genes for ribosomal RNA, and in bacteriophage genes (Dujon *et al.*, 1989; Lambowitz, 1989; Gauthier *et al.*, 1991; Marshall and Lemieux, 1991).

For horizontal transmission of DNA to occur, the genetic material must be routed around at least two hypothetical barriers: transfer barriers that prevent the delivery of genetic information from a donor, and establishment barriers that block inheritance of new molecules (Heinemann and Sprague, 1989; Heinemann, 1991). Some bacterial plasmids and transposons are known to be capable of conjugal transfer among bacterial species and even between bacteria and eukaryotes (Ippen-Ihler and Minkley, 1986; Clewell and Gawron-Burke, 1986; Heinemann, 1991). Conjugation is a reliable method for the transfer and stable maintenance of these mobile genetic elements. Conjugation may also occur in mitochondria. Mitochondrial fusion is also considered to be a basic mechanism for spreading mobile genetic

elements through mitochondrial populations within species against a background of uniparental mtDNA transmission (Zinn and Butow, 1985; Boynton *et al.*, 1987; Remacle *et al.*, 1990).

IV. Mitochondrial Fusion in *Physarum polycephalum*

A. Mitochondrial Life Cycle

1. Life Cycle of *Physarum polycephalum*

Recombination between mitochondrial genomes has also been found in the mitochondrial crosses of a few other organisms, the best studies of which have been with the yeast *Saccharomyces cerevisiae* (Table I). In attempts to solve problems between mitochondrial fusion and recombination, the true slime mold, *Physarum polycephalum,* has been found to serve as an extremely useful experimental system because of its controllable life cycle and characteristic mitochondria (Alexopoulos, 1982; Burland *et al.*, 1993). The life history of *P. polycephalum* is that of a typical haplodiplont, with two distinct stages: the diploid syncytial plasmodium and the haploid uninucleate myxamebas (Fig. 1). Furthermore, the complete life history involves resting stages, as represented by spores, spherules, sclerotia, and cysts. The thick-walled, resistant spores hatch to release myxamebas, which act as isogametes; individual isogametes of different mating types pair to form diploid zygotes. The zygotes develop into giant, diploid syncytia called plasmodia, which may reversibly transform themselves into resistant sclerotia (synonymous with spherules) or may undergo a terminal differentiation process that leads to the formation of lobed sporangia as a result of starvation and illumination. Meiosis occurs within the spores.

The mitochondria of *P. polycephalum* also provide a particularly favorable model for the analysis of division, segregation, and fusion of mt-nuclei. They contain about ten times more mtDNA than mitochondria from other sources. The bulk of the mtDNA is usually packed into an electron-dense mt-nucleus together with RNA and proteins. The mtDNA content per mt-nucleus is estimated to be about 32 molecules, each of which is linear, with a molecular weight of 45×10^6 per mitochondrion at the mG1 phase (Kuroiwa, 1982). Since the mtDNA molecules in *P. polycephalum* are homogeneous in terms of their physical structure, the mt-nucleus seems to be polyploid or polytene (Kawano *et al.*, 1982; Suzuki *et al.*, 1982; Takano *et al.*, 1990). This mt-nucleus facilitates observations of the processes of mt-nuclear duplication, division, and fusion, as well as monitoring the behavior of mtDNA by light and electron microscopy. Using *P. polycephalum,*

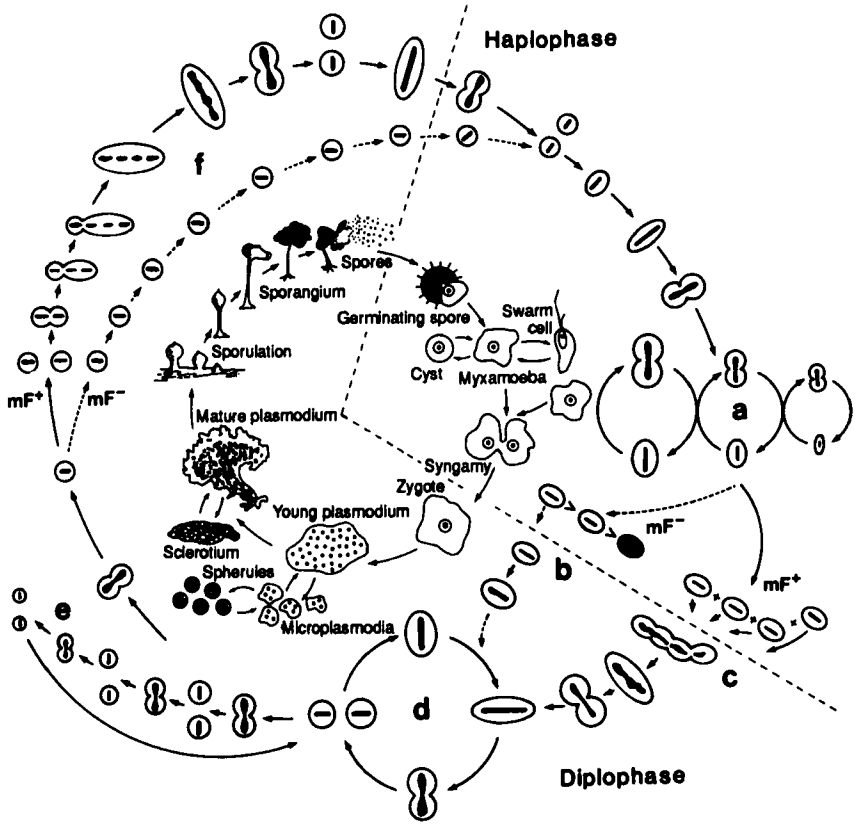


FIG. 1 The life cycle of mitochondria (outer scheme) throughout the life history of *P. polycephalum* (inner scheme). Small spherical, oval, and dumbbell-shaped figures in the outer scheme represent mitochondrial bodies and mitochondrial nuclei. (a) Mitochondrial division cycle and stepwise reduction and rise in volume of mitochondria and mtDNA content of mitochondria nonsynchronizing with mtDNA synthesis through the culture of myxamebas. (b) Hierarchical uniparental transmission of mtDNA during the formation of plasmodium by crossing between mF-strains. (c) Mitochondrial fusion accompanying mt-nuclear fusion promoted by the mF plasmid during formation of plasmodium by crossing between mF^- and mF^+ strains. (d) Mitochondrial division cycle synchronizing with mtDNA synthesis in the plasmodium. (e) Stepwise reduction of mitochondria by the division of mitochondria without DNA synthesis during spherulation. (f) Behavior of mitochondria during sporulation, mitochondrial fusion (Mif^+) carrying the mF plasmid, and mitochondrial fusion-deficient (Mif^-) strain without the mF plasmid. (Modified from Kawano, 1991; Kuroiwa *et al.*, 1994.) From Kuroiwa, T., Ohta, T., Kuroiwa, H., and Kawano, S. (1994). Molecular and cellular mechanisms of mitochondrial nuclear division and mitochondriokinesis. *Microsc. Res. Technol.* **27,** 220–232. Copyright © 1994 John Wiley and Sons. Reprinted by permission of John Wiley & Sons, Inc.

we have studied various cytoplasmic aspects of mitochondrial biogenesis, following the mitochondrial life cycle throughout its history, as shown in Fig. 1 (Kuroiwa, 1982; Kawano, 1991; Kuroiwa *et al.*, 1994). In particular, in this chapter, we focus on and discuss the behavior of the mitochondrial genome throughout the mitochondrial life cycle, the way that fusion and recombination occur, and how the mitochondrial genome is transmitted to the next generation.

2. Mitochondrial Differentiation during Sclerotization and Sporulation

A model system for studies of mitochondrial differentiation should have a uniform population of cells and controllable cellular differentiation. Furthermore, it must be available in sufficient quantities for isolation and characterization of mitochondria. These criteria are satisfied by the period of transition from plasmodia to sclerotization and sporulation in *P. polycephalum*. Under certain adverse conditions (e.g., desiccation, cold temperature, osmotic pressure, starvation, and "aging"), a plasmodium divides up into small sclerotia (spherules), each containing several cell nuclei (Raub and Aldrich, 1982). Walls are established between these spherules and the whole mass appears cellular. The spherules are resistant structures, and they may play an important role in the survival of the myxomycete. During the very early stage of sclerotization, by starvation and/or aging of the culture, sporulation is induced by regulated illumination. Light is an indispensable requirement for fruiting of pigmented plasmodia (Wheals, 1970).

Microplasmodia differentiate into spherules when cultures are aged for 8–10 days. Rates of respiration in the microplasmodia decrease rapidly with aging to give a 90% decrease in the consumption of oxygen over the course of 9 days. These phenomena were examined by isolating and characterizing mitochondria from microplasmodia and spherules at different stages of spherulation (Kawano *et al.*, 1983). Uptake of oxygen by the isolated mitochondria decreased according to spherulation. Mitochondrial differentiation to an inactive state is characterized by a decrease not only in their dimensions but also in their DNA, RNA, and protein contents. Diminutive mitochondria contain small particle-shaped mt-nuclei. The DNA content, measured by microscopic fluorometry, is about 1.15 or 0.58×10^{-10} g, which corresponds to about 16 or 8 copies of the genome, respectively. Finally, the number of copies of the genome per mitochondrion in the completed spherule equilibrated 4 or 2, namely, 1/8 to 1/16 of the original level in the plasmodium (e.g., 32 copies of the genome per mitochondrion at the mG1 phase). The changes in ploidy of the mt-nucleus during spherulation are due to stepwise reduction in the number of whole

mitochondrial genomes as a result of cycles of mitochondrial division without any accompanying mtDNA synthesis.

Recently, a similar phenomenon of the mitochondrial division not being synchronized with mtDNA synthesis has been observed in a culture of myxamebas (Sasaki *et al.*, 1994). A stepwise reduction in the volume and DNA content of organelles has also been observed in plastids of spermatocytes during spermatogenesis in the fern *Pteris vittata* (Kuroiwa *et al.*, 1988), the green algae *Bryopsis maxima* (Kuroiwa and Hori, 1986) and *Chara*

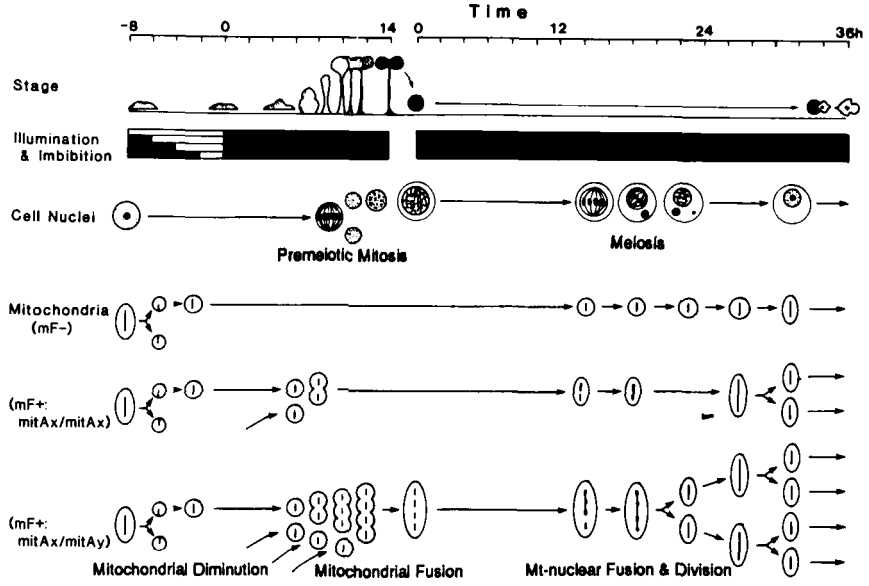
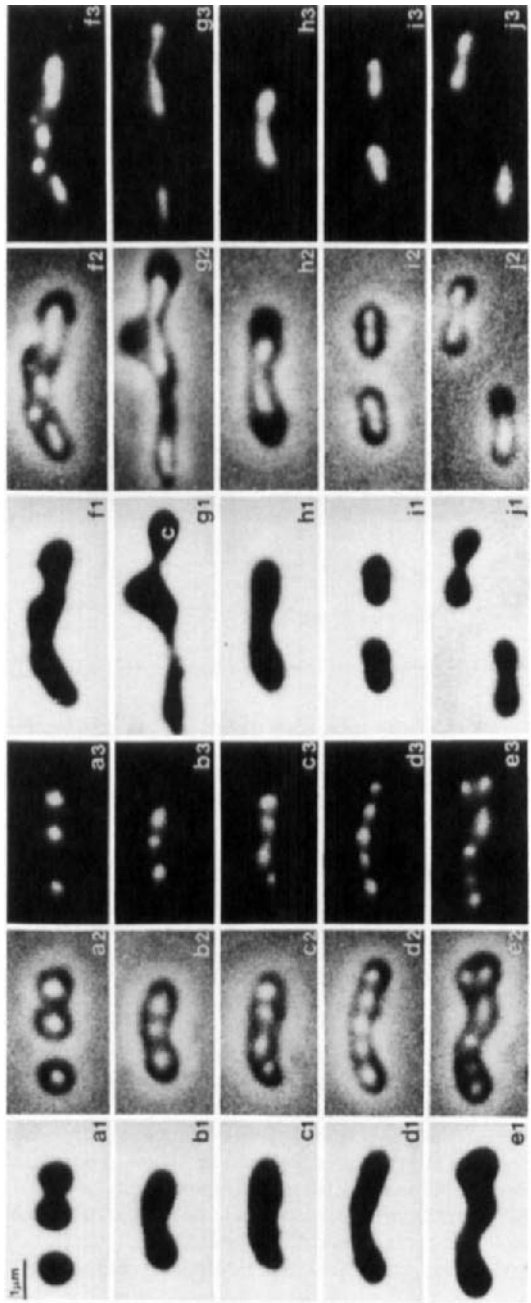


FIG. 2 Diagrammatic representation of morphological changes occurring during sporulation: behavior of cell nuclei, mitochondria and mt-nuclei during sporulation, and spore germination of *Physarum polycephalum*. Sporulation is synchronously started by a light turned off after 2–8 hr of illumination, and germination of spores almost synchronously by imbibition. Cytoplasmic cleavage for spore formation occurs around each cell nucleus after premeitotic mitosis. Cell nuclei enter into the early prophase of meiosis during head blackening of sporangia and become dormant at this stage within spores. The rest of the meiotic processes occur during germination within spores. Mitochondria divide to be spherical or oval during the starved plasmodium stage. Mitochondria fusion starts with sporangium formation in the plasmodial strain carrying the mF plasmid (mF^+) while it fails to do so (<0.7%) in the plasmodial strain without the mF plasmid (mF^-). The fused mitochondria enter into spores, and extensive mt-nuclear fusion and several successive mitochondrial divisions occur during spore germination. The frequency of mitochondrial fusion is controlled by a cell-nuclear locus, *mitA*. Mitochondrial fusion occurs at high frequency (59.2–80.5%) with the combination of unlike alleles (*mitAx/mitAy*) but at intermediate frequency with the combination of like alleles (*mitAx/mitAx*).


1μm
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corallina (Sun *et al.*, 1988), and in plastids during the life cycle of the unicellular alga *Cyanidium caldarium* M-8 (Kuroiwa *et al.*, 1989). These results indicate that there are successive cycles of division of organelles without any concomitant duplication of DNA during the differentiation of cells and gametogenesis in some organisms.

3. Mitochondrial Fusion during Sporulation and Spore Germination

Sporulation of *P. polycephalum* occurs with a high degree of synchrony, and can be easily induced by 4–6 hr of illumination of starved plasmodia. About 8 hr after illumination, many projections form along the plasmodial strand; these increase in size and develop into sporangia over the subsequent 6 hr (Fig. 2). Nishibayashi *et al.* (1987) analyzed the behavior of cell nuclei, mitochondria, and mt-nuclei during sporulation, during which starved plasmodia differentiate into sporangia. The mitochondria also decrease in size between early and midstages of starved plasmodia to differentiate into sporangia as well as sclerotia. The mt-nuclear DNA content per mitochondrion is constant in sclerotia and during the early starved plasmodium stage, but decreases by one half at the midstarved plasmodium stage, and remains the same from the midstarved plasmodium stage to the premeiotic division stage. This process is similar to that during spherulation. At the resting stage of sporulation, dumbbell-shaped or multinucleate mitochondria which contain two or more discrete mt-nuclei frequently appear. The total fluorescence intensity of a few mt-nuclei in multinucleate mitochondria increases stepwise with the number of mt-nuclei. These results suggest that a few mt-nuclei in multinucleate mitochondria partially attach or fuse during the mature stage.

A more detailed study was carried out to determine the events leading to the appearance and subsequent disappearance of multinucleate mitochondria (Kawano *et al.*, 1991a). Mitochondrial fusion occurs at high fre-

FIG. 3 Representative stages in mitochondrial fusion (a–f) during sporulation and division (g–j) during spore germination in *Physarum polycephalum*. Phase-contrast (1), phase-contrast fluorescence (2), and DAPI-fluorescence (3) micrographs are shown for the same field. The mt-nuclei are visible as intense blue spots in the mitochondria by DAPI staining. Small spherical mitochondria fuse with one another to form large, knotted, multinucleate mitochondria (a–e) which subsequently undergo fusion between the mt-nuclei derived from individual mitochondria (f). Mitochondria showing various extents of mitochondrial fusion are enclosed in spores. Several successive mitochondrial divisions follow, accompanied by mt-nuclear divisions (g–j), during spore germination. Such various types of multinucleate mitochondria are also observed in zygotes. Bar = 1 μm. (From Kawano *et al.*, 1991a.)

quency during a limited period of the late sporulation stage (Fig. 3). The small, spherical mitochondria fuse with one another to form dumbbell shapes and other irregular forms (Fig. 3,a) which sometimes take on a rounded shape. Further fusions with uninucleate (Fig. 3,b and c) and multinucleate mitochondria result, by the late resting stage, in the formation of large mitochondria of irregular form (Fig. 3,d and e). Some fusions between mt-nuclei are observed at this stage, and mitochondria showing various extents of mitochondrial and mt-nuclear fusion are enclosed within the spores. Further events in the spores were investigated by suspending mature spores in a drop of distilled water on a microscope slide. Meiosis resumes and is completed 14–18 hr after wetting. Each spore then hatches to yield a single myxameba or swarm cell. When chromosome condensation occurs at the resumption of meiosis, vigorous morphological changes also begin in the multinucleate mitochondria. Irregular-shaped mitochondria undergo successive rounds of unequal division until the daughter mitochondria are similar in size to prefusion mitochondria (Fig. 3, g–j). These mitochondrial divisions are completed by the time of spore hatching.

4. Isolation of Mitochondrial Fusion and Its Defective Strains

P. polycephalum provides particularly favorable material for the analysis of mitochondrial and mt-nuclear fusions. These fusions result in the formation of multinucleate mitochondria in the spore. The mitochondria in spores of many laboratory strains of *P. polycephalum* that were derived from crosses of several different natural isolates were examined by cracking open spores in the presence of glutaraldehyde so that the mitochondria were fixed immediately and retained their normal shape (Fig. 4; Kawano *et al.*, 1993). DAPI-fluorescence microscopy revealed that the spores of strain Ng and its derivatives contained multinucleate mitochondria, as expected from our previous studies (Nishibayashi *et al.*, 1987; Kawano *et al.*, 1991a). Unexpectedly, however, the spores of all the other strains were found to contain only uninucleate mitochondria, suggesting that sporulation in most strains is not accompanied by mitochondrial fusion, which produces the multinucleate state. Spores with uninucleate mitochondria each contain approximately 22–30 small, spherical mitochondria, in which the mitochondrial nuclei are visible as intense blue spots after staining with DAPI (Fig. 4,a–c). By contrast, multinucleate mitochondria are large and irregular in shape, containing several mitochondrial nuclei, with more mitochondrial nuclei in large mitochondria. Spores containing multinucleate mitochondria were classified as being of two types. One type contained approximately 12–16 mitochondria, and somewhat more than half of these mitochondria were large, irregular in shape, and multinucleate (Fig. 4,d–f). The other type contained only a few multinucleate mitochondria (Fig. 4,g–i). Mitochon-

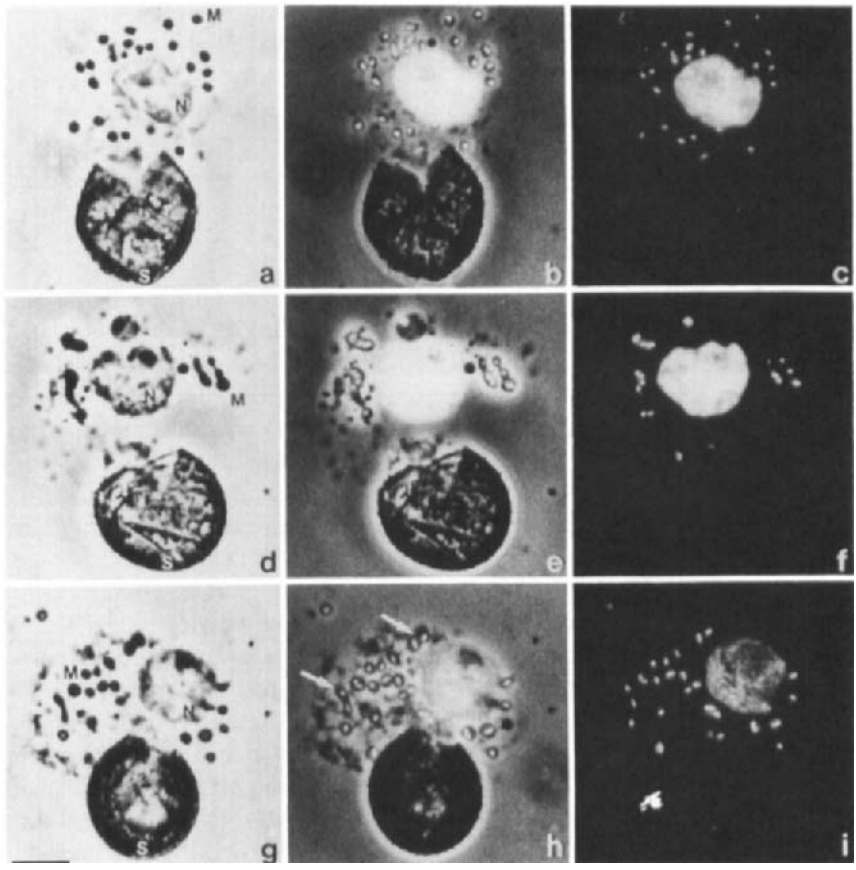


FIG. 4 Mif⁻ (mitochondrial fusion-deficient; a–c) and Mif⁺ (mitochondrial fusion; d–i) phenotypes of the spores cracked open in *Physarum polycephalum*. Phase-contrast (a, d, and g), phase-contrast fluorescence (b, e, and h), and DAPI-fluorescence (c, f, and i) micrographs are shown for the same field. The mt-nuclei are visible as intense blue spots in the mitochondria as a result of staining with DAPI. The Mif⁺ phenotype was classified as high-frequency (d–f) and low-frequency (g–i) according to the frequency of multinucleate mitochondria. M, mitochondrion; N, nucleus; S, spore wall. Bar = 5 μm. (From Kawano *et al.*, 1993.)

drial fusion occurs frequently during the late stages of sporulation. The small, spherical mitochondria fuse with one another to form dumbbells and various other irregular shapes that sometimes tend toward spherical. Further fusions of uninucleate and multinucleate mitochondria result in the formation of large mitochondria of irregular shape, and mitochondria showing evidence of mithcondrial fusion to various extents were enclosed

within the spores. The frequency of multinucleate mitochondria in spores seems to reflect the frequency of mitochondrial fusions.

B. Genetic Analysis of Mitochondrial Fusion

1. Hierarchical Transmission of mtDNA during Plasmodium Formation

In *P. polycephalum,* haploid myxamebas act as isogametes, fusing in pairs of individuals of different mating types to form diploid zygotes which develop into macroscopic, diploid plasmodia by repeated mitotic cycles in the absence of cell division (Fig. 1). Thus, the random partitioning of mitochondria by cell division does not occur during formation of the plasmodium. Attempts have been made to determine whether a heteroplasmic zygote produced by mating would develop into a homoplasmic plasmodium in spite of the absence of such random partitioning. However, the inheritance of mitochondrial genes in *P. polycephalum* is difficult to study because easily scored cytoplasmic mutants have not been available. Therefore, the analysis with restriction endonucleases was performed with mtDNA from 19 plasmodial strains (Kawano *et al.,* 1987a). The extent of variation in mtDNA among these strains is high in comparison to that found in other organisms, and it provides a useful source of cytoplasmic genetic markers. Although plasmodia of *P. polycephalum* are diploid, being formed by fusion of myxamebal isogametes, each of the 19 plasmodia possesses mtDNA of only a single type. The pattern of transmission of mtDNA during formation of the plasmodium was studied by mating pairs of myxamebal strains with mtDNAs of different types (Kawano *et al.,* 1987b). Transmission is uniparental; the plasmodia that were formed carried mtDNA with the restriction pattern of only one of the two parental types. Since diploid zygotes develop into plasmodia by repeated mitotic cycles in the absence of cell division, it is clear that this uniparental transmission of mtDNA does not depend upon random partitioning either of the mitochondria or of the mtDNA molecules during cell division.

The mating-type system of *P. polycephalum* appears particularly complex and it is, therefore, of some interest to determine the way in which mitochondrial inheritance is regulated in this organism (Fig. 5). The sexual development (crossing) is under the control of a mating-type system which consists of three loci: *matA, matB,* and *matC* (Dee, 1966; Youngman *et al.,* 1979; Kirouac-Brunet *et al.,* 1981; Kawano *et al.,* 1987a; Dee, 1982, 1987). To cross efficiently, myxamebas must carry different alleles of at least *matA* and *matB.* For each of these loci, full compatibility results when any two

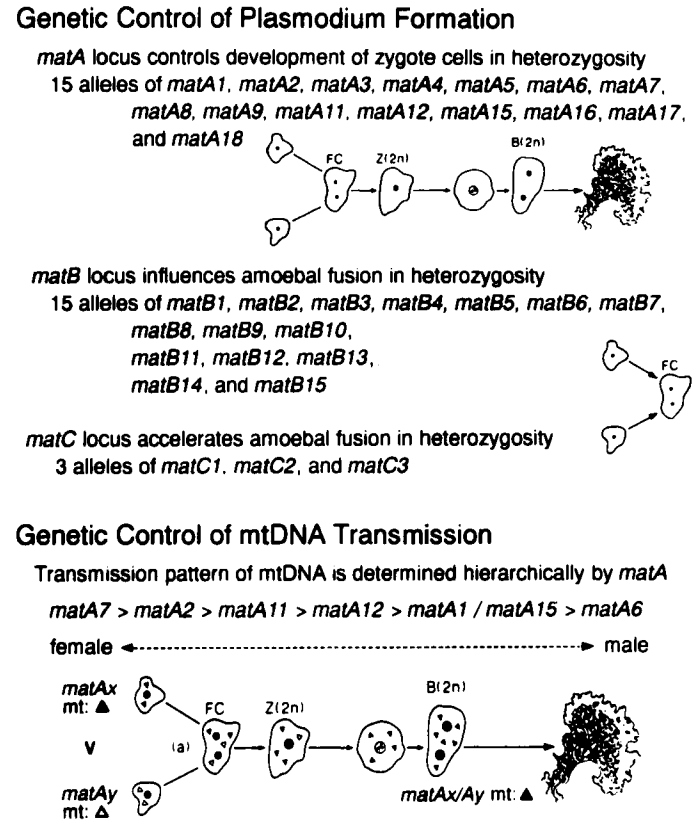


FIG. 5 Mating loci and hierarchical transmission pattern of mtDNA in *Physarum polycephalum*. Effects and alleles of *matA*, *matB*, and *matC* are shown. A fusion cell (FC) is heteroallelic for *matA:* plasmodium development is initiated, nuclear fusion occurs in interphase, and the zygote (Z) undergoes plasmodial mitosis without cytokinesis to a plasmodium via a binucleate cell (B). FC is homoallelic for *matA:* cells remain ameboid and may separate again. The *matB* and *matC* both regulate cell fusion, apparently by promoting the probability of cell fusion in heterozygosity. The transmission pattern of mtDNA is uniparental: the mtDNA donor in any cross would be the strain of higher status in the order of *matA* represented by a reduced hierarchy (note that *matA1* and *matB15* have not been tested against each other).

alleles are combined from among a set of at least thirteen. In addition, heteroallelism is necessary for *matC*, which has at least three alleles, when crosses are carried out under conditions of elevated pH or reduced ionic strength (Shinnick *et al.*, 1978; Kawano *et al.*, 1987a). As described above, uinparental inheritance of mtDNA is demonstrated in crosses that involved one particular pair of strains. Thus, there may be a consistent bias in favor

of the mtDNA from one of these strains. If so, it is clearly necessary to investigate whether such bias could be correlated with particular mitochondrial genomes or myxamebal mating types.

Plasmodia were generated by matings between pairs of myxamebal strains that carried mtDNA molecules distinguishable by restriction endonuclease digestion (Kawano and Kuroiwa, 1989). In each mating pair, one strain consistently acted as the donor of mtDNA, but this strain does not always act as the mtDNA donor when combined in other mating pairs. The identity of the mtDNA donor in each pair is not determined by the different types of mtDNA molecule present or by the different alleles of *matB* or *matC,* two mating-type loci that regulate myxamebal fusion. The results suggest that alleles of the third mating-type locus, *matA,* which controls zygote development, might form a hierarchy such that the mtDNA donor in any cross would be the strain of higher status (Fig. 5). The deduced hierarchy is *matA2* > *matA11* > *matA12* > *matA1.* Recently, such a hierarchy has been confirmed independently by Meland *et al.* (1991). Pooling our data with those of theirs, the tentative order of the *matA* hierarchy with regard to the inheritance of mtDNA is as follows: *matA7* > *matA2* > *matA11* > *matA12* > *matA1/matA15* > *matA6* (note that *matA1* and *matA15* have not been tested against each other).

Meland *et al.* (1991) have also shown that one parental mitochondrial genome is rapidly eliminated from the plasmodium formed when two myxameba fuse and give rise to a zygote which subsequently develops into the plasmodium. Although it is not possible to rigorously exclude "outreplication," their data seem to be most consistent with the "active degradation" model. The quantitative analysis which overestimates the percentage of the "recessive" genomes present during differentiation suggests that this likely process of active degradation is initiated immediately after gamete fusion and is virtually completed by the time of the second division in the newly differentiated plasmodium.

Active degradation of organelle genomes has been proposed for plastids. An active process is indicated during sexual crosses of some isogamous green algae (Kuroiwa, 1985) and during senescence and gametogenesis in higher plants (Sun *et al.,* 1988; Sodmergen *et al.,* 1989, 1991, 1992; Kuroiwa *et al.,* 1991b). The best-studied example is the uniparental inheritance of the chloroplast genome in *Chlamydomonas reinhardtii.* One parental chloroplast genome disappears in young zygotes during the first 6 hr after mating (Kuroiwa *et al.,* 1982). Two different hypotheses have been proposed to explain this rapid elimination: the "methylation-restriction hypothesis" (Sager and Gabowy, 1985) and the "active digestion hypothesis" (Kuroiwa, 1985). The chloroplast inheritance in *C. reinhardtii* is closely related to the mating-type system. This is analogous to the situation of mtDNA in *P. polycephalum.* However, in this case, the mechanism may be much more

complex since at least 13 alleles of the *matA* locus are known, compared with only two in *C. reinhardtii.* It is of interest to note that in *P. polycephalum* there is precedence for a genomic restriction system. After normal fusion of genetically compatible plasmodia (Poulter and Dee, 1968), the strain carrying dominant *kil/let* alleles apparently causes destruction of nuclei from the strain carrying recessive alleles. The nuclei of the sensitive strain are selectively destroyed, enclosed in vacuoles, and eliminated from the cytoplasm. There is no evidence that the *matA* alleles are involved in this process; nor is there any evidence for a hierarchical system of elimination. Nevertheless, there are analogies worth keeping in mind in the continuing analysis of the mtDNA inheritance system, as described by Meland *et al.* (1991).

2. A Mitochondrial Genetic Element Controlling Mitochondrial Fusion

Two distinct mitochondrial phenotypes, Mif^+ and Mif^-, are observed in the spores of *P. polycephalum* as described earlier (Fig. 4). The pattern of transmission of the Mif character was studied in a series of crosses between myxamebal strains (Kawano *et al.,* 1991a, 1993). The results can be explained by postulating that the occurrence of mitochondrial fusion in a plasmodium depends upon the presence of a single factor (mif^+) which is transmitted to most or all of the myxamebal progeny of a Mif^+ plasmodium. The mif^+ factor is apparently present in the original natural isolate from which the plasmodial strain Ng was derived and is passed on to mif^+ myxamebal strains (NG7 and OZ strains). All other isolates had the mif^- genotype (Kawano *et al.,* 1991b), for example, the myxamebal strains (e.g., OX110 and OX115) that are largely isogenic with the Colonia isolate (Kawano *et al.,* 1991b); both are classified as mif^- because plasmodia from their crosses with other mif^- strains are of the Mif^- phenotype and yield myxamebal progeny that all formed Mif^- plasmodia when mated with other mif^- strains. By contrast, NG7 was classified as mif^+ because it always gave rise to Mif^+ plasmodia, even when mated with a mif^- strain, and the progeny of these plasmodia always transmitted the Mif^+ character. Such preferential transmission of the mif^+ factor has been demonstrated over five successive sexual generations in which progeny of Mif^+ plasmodia were mated with mif^- myxamebae.

This suggests that Mif^+ may be of mitochondrial rather than nuclear origin. The mtDNA of appropriate myxamebal and plasmodial strains was, therefore, analyzed by agarose gel electrophoresis (Kawano *et al.,* 1991a, 1993). Figure 6 shows the patterns of *Hin*dIII restriction fragments of mtDNA from twenty representative strains. Twenty distinct types of mtDNA were detected. The patterns show the conservation of certain

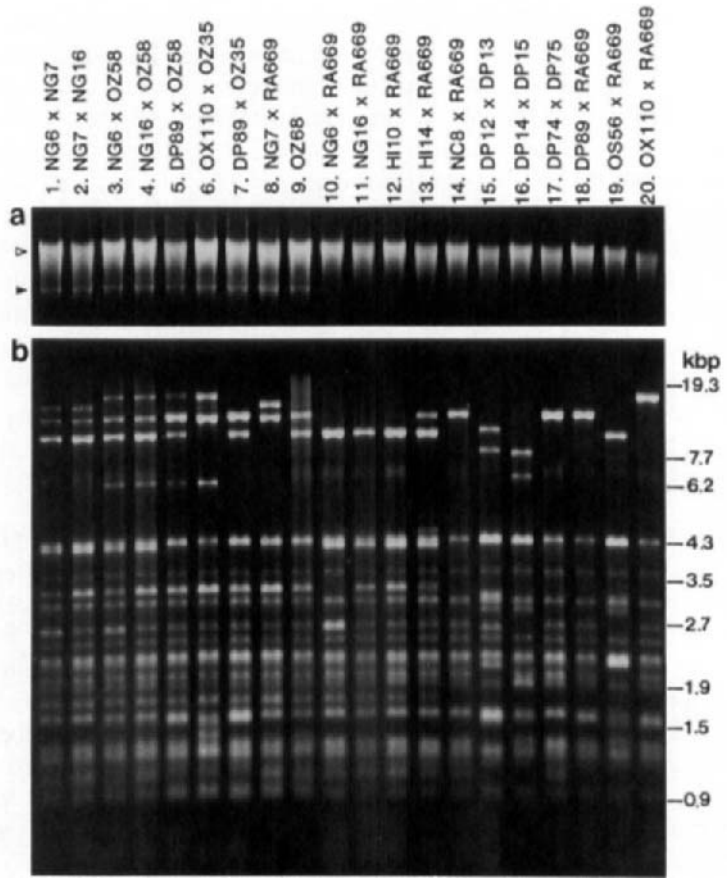


FIG. 6 Profiles after electrophoresis on an agarose gel of undigested (a) and *Hin*dIII-digested (b) mtDNAs from 20 plasmodial Mif$^+$ (lanes 1–9) and Mif$^-$ (lanes 10–20) strains. All Mif$^+$ strains carry the mF plasmid (mF$^+$) while all Mif$^-$ do not carry the mF plasmid (mF$^-$). Open and closed arrowheads in (a) indicate bands of mtDNA and mF plasmid, respectively. The *Hin*dIII restriction patterns indicate that high-frequency mitochondrial fusion types (lanes 1–5) are heteroplasmic, while intermediate frequency mitochondrial fusion types (lanes 6–9) and Mif$^-$ strains are homoplasmic in these profiles. (From Kawano *et al.*, 1993.)

restriction fragments between the different types of mtDNA, but fragments specific for the Mif$^+$ strains cannot be detected at first glance. However, from agarose gel electrophoresis of undigested mtDNA (Fig. 6a), the mtDNAs were classified into two types according to their origin in Mif$^+$ or Mif$^-$ strains. The mtDNA of each Mif$^-$ strain formed a single band of 80–90 kbp. In addition to this main band, Mif$^+$ strains always gave a second band of approxiamtely 12–16 kbp. The molar ratio of the main mitochon-

drial genome to the plasmid was estimated to be roughly constant from the gel profiles. These results suggest that the *mif*$^+$ character might be carried on this plasmid DNA.

Electrophoretic analysis with the restriction endonuclease *Hin*dIII also shows that there is a direct relationship between mitochondrial fusion and reorganization of mtDNA (Fig. 7B). As described earlier, an analysis of restriction fragment length polymorphisms (RFLPs) shows that only one of the two myxamebal strains in a mating culture transmits its mtDNA to the plasmodia. These earlier studies all involved *mif*$^-$ × *mif*$^-$ matings (see Fig. 7A for an example of uniparental inheritance in such a mating). In contrast, results of *mif*$^-$ × *mif*$^+$ matings do not show simple uniparental inheritance of mtDNA; mtDNA from the plasmodia formed from such matings shows some restriction fragments characteristic of the plasmid DNA and the mtDNA, as well as two fragments of novel sizes (11.0 kbp and 6.5 kbp, Fig. 7B). This result shows that reorganization of the mtDNA is promoted by the mitochondrial and mt-nuclear fusions. Nevertheless, the pattern of restriction fragments from the plasmid DNA remained unchanged over at least five successive sexual generations derived from *mif*$^-$ × *mif*$^+$ matings, as shown by the restriction pattern of the plasmid DNA purified from agarose gel (Fig. 7C).

These results suggest that the *mif*$^+$ character is carried on the 16-kbp plasmid DNA. Moreover, Southern hybridizations with labeled plasmid DNA as the probe demonstrate that some plasmid DNA sequences are represented in the reorganized mitochondrial genomes of the plasmodia generated by the *mif*$^-$ × *mif*$^+$ mating. To our surprise, not only in the case of all *mif*$^+$ strains but also in the case of the *mif*$^-$ strains (OX110 and NG6), the labeled plasmid probe hybridizes to bands of the mitochondrial genome (Figs. 6 and 7). The mtDNA of all myxamebal strains has been confirmed to have a duplicated region that contains certain sequences homologous to the plasmid (Section V), visible as a double-bright 15.5-kbp *Hin*d III fragment. On the other hand, NG6 is unusual in that it is one of three *mif*$^-$ myxamebal offspring of 50 analyzed from an Ng Mif$^+$ plasmodium. More detailed information on the extent of homology between the plasmid DNA and the mitochondrial genome of NG6 is obtained by restriction endonuclease digestion of mtDNAs and further hybridization analysis (lanes 2 and 5 in Fig. 7). Only part of the sequence of the plasmid DNA was present in NG6, compared with the inbred *mif*$^+$ strain NG7 (lanes 2 and 8 in Fig. 7B). This result suggests that the sequences deleted in NG6 are probably necessary for *mif*$^+$ expression. On the other hand, the two fragments (11.0 kbp and 6.5 kbp) newly generated as a result of the reorganization of mtDNA hybridized strongly with labeled plasmid DNA used as the probe (lanes 10 and 11 in Fig. 7B). This result showed that the mtDNA was reorganized by insertion of certain sequences of the plasmid (see Section V).

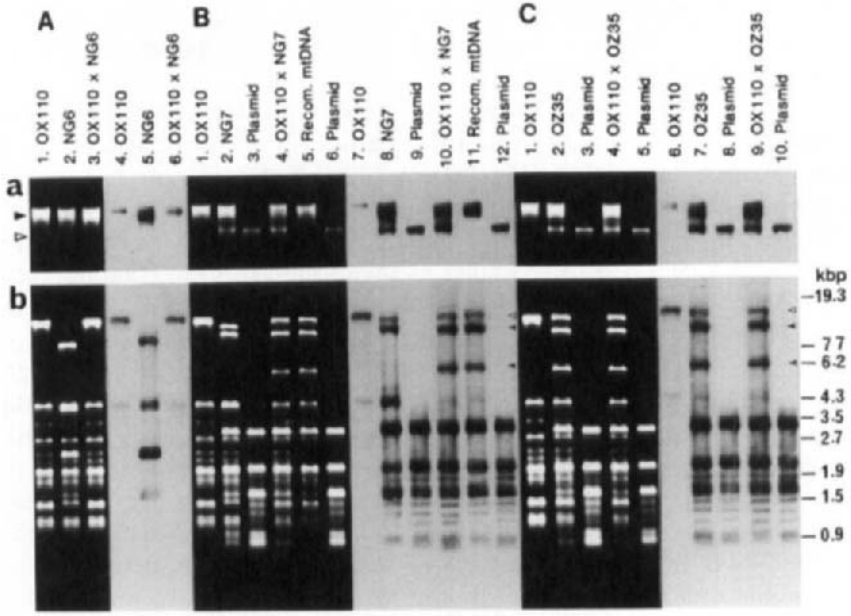


FIG. 7 Transmission of mtDNA in matings between myxamebas. (a) Undigested mtDNA. (b) mtDNA digested with *Hin*dIII. Lanes 1–3 in panel A, lanes 1–6 in panel B, and lanes 1–5 in panel C show the results of electrophoresis on 1% agarose gels; lanes 4–6 in panel A, lanes 7–12 in panel B, and lanes 6–10 in panel C show Southern hybridization patterns of the same gels using labeled plasmid DNA as the probe. (A) The mtDNAs of mF^- myxamebal strains (lanes 1 and 4, OX110; lanes 2 and 5, NG6) and the mating product, Mif^- plasmodium (lanes 3 and 6, OX110 × NG6). (B) mF^- (lanes 1 and 7, OX110) and mF^+ (lanes 2 and 8, NG7) strains; the mating product, Mif^+ plasmodium (lanes 4 and 10, OX110 × NG7); mF plasmid DNAs purified from NG7 and from OX110 × NG7 (lanes 3 and 9, lanes 6 and 12) and recombinant mtDNA purified from OX110 × NG7 (lanes 5 and 11), respectively. (C) mF^- (lanes 1 and 6, OX110) and mF^+ (lanes 2 and 7, OZ35); the mating product, Mif^+ plasmodium (lanes 4 and 9, OX110 × OZ35); mF plasmid DNAs purified from mF^+ myxamebal and Mif^+ plasmodial OX110 × OZ35 (lanes 3 and 8, lanes 5 and 10). Open and closed large arrowheads in (a) indicate bands of mtDNA and plasmids, respectively. Open and closed small arrowheads in panels B and C indicate the fragment (15.5 kbp) of mtDNA before insertion of the plasmid and the newly formed fragments (11.0 and 6.5 kbp) that resulted from insertion of the plasmid, respectively.

3. Nuclear Alleles Controlling the Frequency of Mitochondrial Fusion

The Mif^+ strains frequently have multinucleate mitochondria in their spores indicative of mitochondrial fusion, but these frequencies can be classified into two types: high multinucleate (59.2%–80.5%) and intermediate multinucleate (12.8%–21.3%) (Fig. 4). The Mif^- strains and their progeny have

very low frequencies (<0.7%) of multinucleate mitochondria at all stages of the life cycle, and no additional mitochondrial fusion occurs during sporulation in the Mif$^-$ strains. Such very low frequencies (0.1%–0.7%) of multinucleate mitochondria in spores are here designated as no mitochondrial fusion, although they all exhibit finite levels. The restriction patterns of the mtDNA of the intermediate frequency spore type indicate that they are homoplasmic: within each plasmodium, mtDNA of only a single, parental type is present. The patterns of the high-frequency type suggest that in every case they are heteroplasmic: within each plasmodium, mtDNAs of both parental types are present. The possibility that the high-frequency mitochondrial fusion type will more often be heteroplasmic is investigated by mating myxamebal strains with mtDNA of different types having quite distinct restriction patterns. The results of these matings clearly show the link between the heteroplasmic condition and the high frequency of mitochondrial fusion (Kawano *et al.*, 1993).

The high-frequency mitochondrial fusion tends to yield the heteroplasmic condition, with two kinds of mitochondria or mtDNA present, one from each parent, in addition to the mF plasmid. However, this situation does not imply that the frequency of mitochondrial fusion is regulated by the mtDNA itself. The two phenotypes, high-frequency and intermediate-frequency mitochondrial fusion, segregated almost equally among the progeny of these crosses. These results suggest that a nuclear gene locus exists, which controls the frequency of mitochondrial fusion. Mitochondrial fusion occurs at high frequency with a combination of unlike alleles at the locus but at intermediate frequency with a combination of like alleles. These features also suggest a mating-type system that consists of three loci: *matA*, *matB*, and *matC*. To cross efficiently, myxamebas must carry different alleles of at least *matA* and *matB*. Therefore, the alleles that regulate the efficiency of the mitochondrial fusion have been tentatively designated mitochondrial mating-type alleles, *mitA1*, *mitA2*, and *mitA3*. To verify this idea, we isolated new tester strains carrying *mitA1*, *mitA2*, and *mitA3*, and classified the progeny of high-frequency mitochondrial fusion crosses into two classes, each carrying two different *mitA* alleles. However, precise frequencies of mitochondrial fusion observed in these crosses with the tester strains varied widely. This wide variation suggests the possibility that further nuclear or mitochondrial loci that control the efficiency of the mitochondrial fusion are present. To define all the details of the genetic system that controls mitochondrial fusion in *P. polycephalum*, further genetic analysis is clearly necessary. Transmission genetics is of major importance in studies of mitochondrial genetics (Birky, 1978; Birky *et al.*, 1982). Mitochondrial fusion influences the pattern of transmission of mtDNA and the mitochondrial plasmid. Mitochondrial fusion, however, is often ignored in spite of its

obvious importance. Such studies as ours on the genetic system that controls mitochondrial fusion should provide new insight in this field.

V. Mitochondrial Fusion-Promoting Plasmid

A. Structural Features of the mF Plasmid

1. Genetic Organization of the mF Plasmid

The mF plasmid is a linear molecule with telomeric-repeated structures (Fig. 8). The genetic organization of the mF plasmid is interesting because it is the longest one of the mitochondrial linear plasmids and has an obvious phenotype that causes mitochondrial fusion (Table II). To detect ORFs of

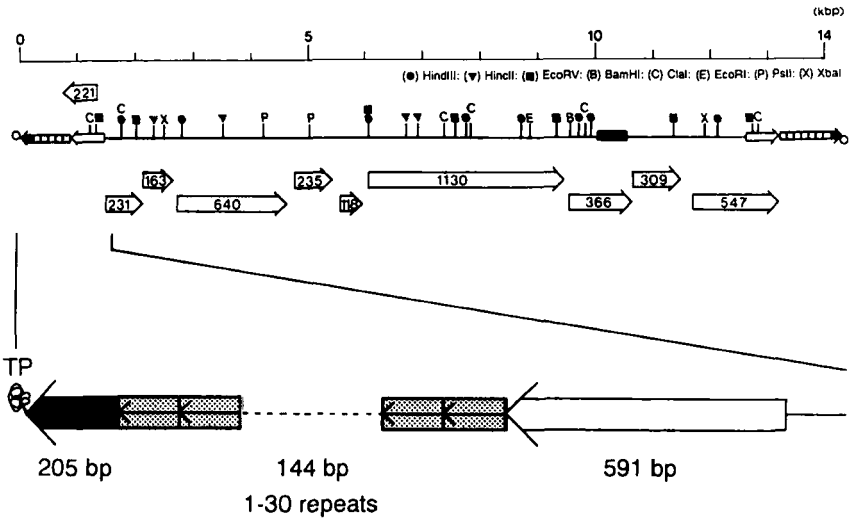


FIG. 8 Structural feature and gene organization of the mF plasmid of *Physarum polycephalum*. Complete nucleotide sequence of mF plasmid including 5 (right) and 7 (left) 144 bp repeating units totaling 14,503 bp. To facilitate the positions of ORFs, a scale and the restriction map of the mF plasmid are shown in the first and second rows, respectively. The locations and orientations of ORFs are indicated by open arrows in the third row. The numbers in open arrows show the number of amino acid residues from ORFs. The terminal structure of the mF plasmid is shown in the fourth row. There is a 205-bp TIR at the extreme ends, which is shown as a closed arrow. The 144-bp repeating units are shown by the shaded boxes. 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. The 5′ end of the mF plasmid is protected by putative terminal proteins (TP). (Modified from Takano *et al.*, 1994a,b.)

the mF plasmid and to determine the gene(s) associated with the mitochondrial fusion, Takano *et al.* (1994b) determined the entire DNA sequence of the mF plasmid. Since the mF plasmid has three TIRs, one of which has 144-bp repeating units, the size of the mF plasmid is estimated to vary from 13.3 to 18.2 kbp, depending on the extent of repetitions. Using certain clones, including 5 and 7 of the 144-bp repeating units for sequencing right and left ends, respectively, the size of the mF plasmid was determined to be 14,503 bp. Overall AT content of the mF plasmid is 75.0%.

The mF plasmid contains 10 ORFs which have potential methionine initiation codons. These ORFs are named according to the number of amino acid residues. All ORFs except one (ORF-221) are encoded on the same strand. The amino acid sequences derived from these ORFs, except ORF-547, do not show significant homology to any amino acid sequences in the database (SWISS-PROT compiled by EMBO). Northern hybridization and primer extension suggest 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. The transcription initiation site (TTATAAG ATATA) locates near the inner end of the left TIR of 591 bp and the transcripts at 1.0, 3.4, and 4.6 knt (kilonucleotide) and longer transcripts start from this transcription initiation site. The 3.5-knt transcripts correspond to the coding region of ORF-1130 and may be derived from the longer transcripts of over 8 knt. Southern hybridization using ^{32}P-labeled mtRNA has shown that the transcripts of the upper region of the coding strand were about 500 times more abundant than those of the lower region (Takano *et al.*, 1994b).

The transcripts of the ORFs of pClK1 and *kalilo* have also been analyzed (Duvell *et al.*, 1988; Gessner-Ulrich and Tudzynski, 1992; Griffiths, 1992). In the case of pClK1, two major transcripts correspond to ORF1 (DNA polymerase) and ORF2 (RNA polymerase). These ORFs start in the TIRs of pClK1 and continue through the inner region. 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 is more complex than that of other such examples and quite different from the other linear mitochondrial plasmids.

2. Terminal Structure of the mF Plasmid

The replicative completion of a linear DNA molecule has been recognized as a serious problem 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 thus result in the progressive loss of the 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 (Meinhardt *et al.*, 1990). By contrast, the terminal structure of the mF plasmid is different from that of other linear plasmids (Figs. 8 and 9; Takano *et al.*, 1991, 1994a).

The mF plasmid has two TIRs 591 and 205 bp long, respectively, which are on either side of one or more 144-bp repeats. The number of 144-bp repeating units varies from one to more than 17 (Fig. 9). This variation results in mF plasmids of different lengths. Repeated arrays at the extreme ends of linear chromosomes are known as telomeres (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, since the 144-bp repeating units of the mF plasmid are not located at the extreme ends, they are quite different from telomeres. The resistance of the ends of mF plasmid to 5′-specific λ exonuclease suggests that the 5′ end may be protected by terminal proteins similar to those found in other linear mitochon-

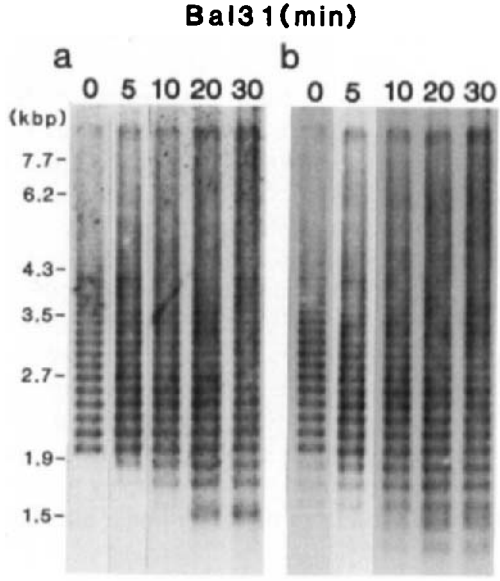


FIG. 9 The repeated structure of the mF plasmid and its sensitivity to exonuclease *Bal*31. The total DNA from mitochondria was digested with *Bal*31 for 0, 5, 10, 20, and 30 min, as indicated. The samples were then digested with *Xba*I and digests were fractionated by agarose-gel electrophoresis. The gel was blotted on a nylon membrane and hybridized with the probes, including the regions very near the left (a) and right (b) ends. The results suggest that the mF plasmid has extensive repeated sequences as a unit of 144 bp at or very near both ends, such as those degraded by exonuclease *Bal*31. The most extensive array of repeats can be estimated to consist of at least 17 repetitions of these repeating units. (From Takano *et al.*, 1991.)

drial 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.

The mechanism that causes the repeating units to occur in varying numbers is still unexplained. The sequence of the last seven 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 bacteriophage 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 intrarecombination 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 has a higher GC content than the other region of the mF plasmid and forms three thermodynamically stable hairpin structures. It may protect against digestion from the ends by exonucleases, and/or recognition of DNA polymerase and a possible terminal protein by its replication system. However, a distinct gene that codes a terminal protein has not been disclosed in the nucleotide sequences of the mF plasmid. Chan *et al.* (1991) and Court and Bertrand (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 composed of consistently spaced serine, tyrosine, lysine, and asparagine residues; they are also present in the terminal proteins of bacteriophages ϕ29 and PRD1, and adenoviruses. 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 is no information about the amino acid sequence of even one terminal protein from a mitochondrial plasmid, nor are there 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.

3. DNA Polymerase Genes

The ORF-547 of the mF plasmid shows extensive homology with the putative DNA polymerases that are encoded on other linear mitochondrial plasmids (Fig. 10). ORFs encoding DNA polymerases have been described in other linear mitochondrial plasmids (Table I). Two conserved domains are characteristic of the proofreading and polymerization motifs of DNA polymerases in linear plasmids and linear phages. The proofreading domain, which has 3′ to 5′ exonuclease activity, is located at the N-terminal 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 10 shows that ORF-547 contains these three polymerization blocks but does not contain the three conserved blocks of the proofreading domain. These results suggest that the DNA polymerase encoded by ORF-547 has DNA polymerizing activity but not the proofreading 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). Two hypotheses exist with regard to the proofreading 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 has exonuclease activity without the exonuclease domain. The

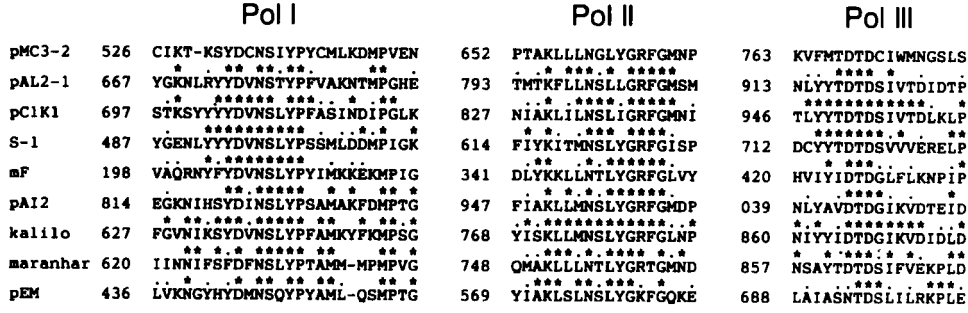


FIG. 10 Polymerizing domains of DNA polymerases coded on ORF-547. The DNA polymerases shown here are as follows: the linear mitochondrial plasmids, pMC3-2 (Rohe *et al.,* 1991), pAL2-1 (Hermanns and Osiewacz, 1992), pC1K1 (Oeser and Tudzynski, 1989), S1 (Kuzumin and Levchenko, 1987), pAI2 (Kempken *et al.,* 1989), *kalilo* (Chan *et al.,* 1991), *maranhar* (Court and Bertrand, 1992), and pEM (Robison *et al.,* 1991). The sequences are 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. (From Takano *et al.,* 1994b.)

other hypothesis is that extensive editing of mRNA creates the 3′ to 5′ exonuclease domain of the DNA polymerase. The extensive editing of the gene for the α subunit of ATP synthetases has been reported in the mitochondria of *P. polycephalum* (Mahendran *et al.*, 1991).

The left end of the mF plasmid does not contain any long ORFs (> 300 amino acids). The longest ORF is encoded in the 591-bp TIR and the 144-bp repeating unit. This ORF (ORF-211) starts in the 591-bp TIR as shown in Fig. 8, if the first ATG codon after the stop codons of the same frame can be considered the initiation codon, and if it encoded a polypeptide of 221 amino acids. Since the 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.

B. Constitutive Recombination Mediated by the mF Plasmid

1. Constitutive Homologous Recombination between the mF Plasmid and mtDNA

To analyze the pattern of transmission of the mtDNA, the novel restriction fragments in $mF^- \times mF^+$ (the 11.0- and 6.5-kbp *Hin*dIII fragments; Fig. 7), which are not found in the restriction pattern of the mtDNA of mF^-, were cloned into the pBluescript vector, and compared with those of the mtDNA and the mF plasmid (Fig. 11; Takano *et al.*, 1992). These fragments consist of one part that is identical to the duplication of the mtDNA (M type) of mF^+ and one part that is identical to the mF plasmid. This result suggests that the novel fragments are generated by recombination between the duplication of the M type mtDNA and the mF plasmid. The recombination occurs 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 seems to be caused by reciprocal crossing-over between the mtDNA of the M type and the mF plasmid.

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. 12; Takano *et al.*, 1992). The mF plasmid has a 475-bp sequence that is identical to a 479-bp sequence of the mtDNA with the exception of deletions of a total of only four base pairs at three sites. The identical sequence of the mtDNA starts from nucleotides 5′TAAAAGAAA 3′ and stops at 5′TTTGTTTG 3′ in the duplication of the mtDNA. By contrast, the identical sequence in the mF plasmid starts at nucleotides 5′ TAAAGAAA 3′ with a one-base deletion and stops at 5′ TTTGTTTG 3′, which is the same as the sequence in the mtDNA. Other deletions in the mF plasmid correspond to positions

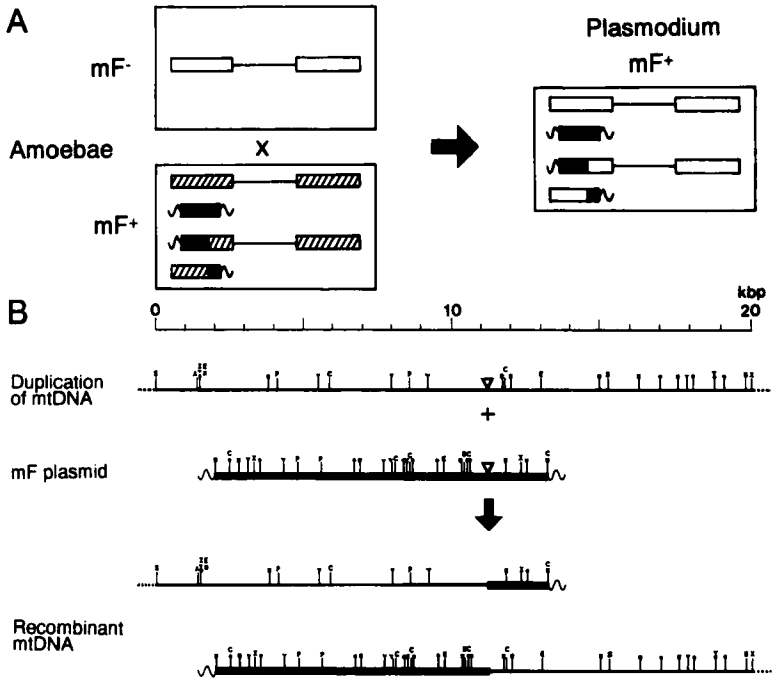


FIG. 11 Transmission patterns (upper part) and constitutive recombination (lower part) of mtDNA and the mF plasmid in the mating between mF^- and mF^+ strains. The mtDNAs of two parents are shown by a thin line (single-copy region) with open or closed boxes (duplication region). In this mating, mtDNA of the mF^- strain is uniparentally transmitted to the resulting plasmodium since the allele of *matA* of the mF^- strain is chosen to be of the female type (mtDNA donor) in the hierarchical order of uniparental transmission as shown in Fig. 5. At this time, a part of the transmitted mtDNA is constitutively recombined with the mF plasmid. Restriction maps of the duplication regions clearly show that the crossing-over between mtDNA and mF plasmid occurs at a specific site (∇) in the duplication regions. The upper thin line and middle thick line show 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 the restriction map of recombinant mtDNA formed by crossing-over between mtDNA and mF plasmid. Thin and thick lines in the restriction map of recombinant mtDNA represent the duplication of mtDNA and mF plasmid, respectively. The wavy line shows the end of the mF plasmid and reflects the heterogeneity in size. The vertical lines with symbols indicate the various restriction sites. (Largely modified from Takano *et al.*, 1992.)

22 and 91–92 of the mtDNA. From these deletion sites, the recombination is estimated to occur at a region between positions 92 and 479 in the identical sequence of the mtDNA type. Such constitutive reciprocal crossing-over between the mtDNA and the mF plasmid caused structural changes in the mtDNA. The mF plasmid crosses over with the duplication of the mtDNA, so that three types of structural conversions of the mtDNA are possible:

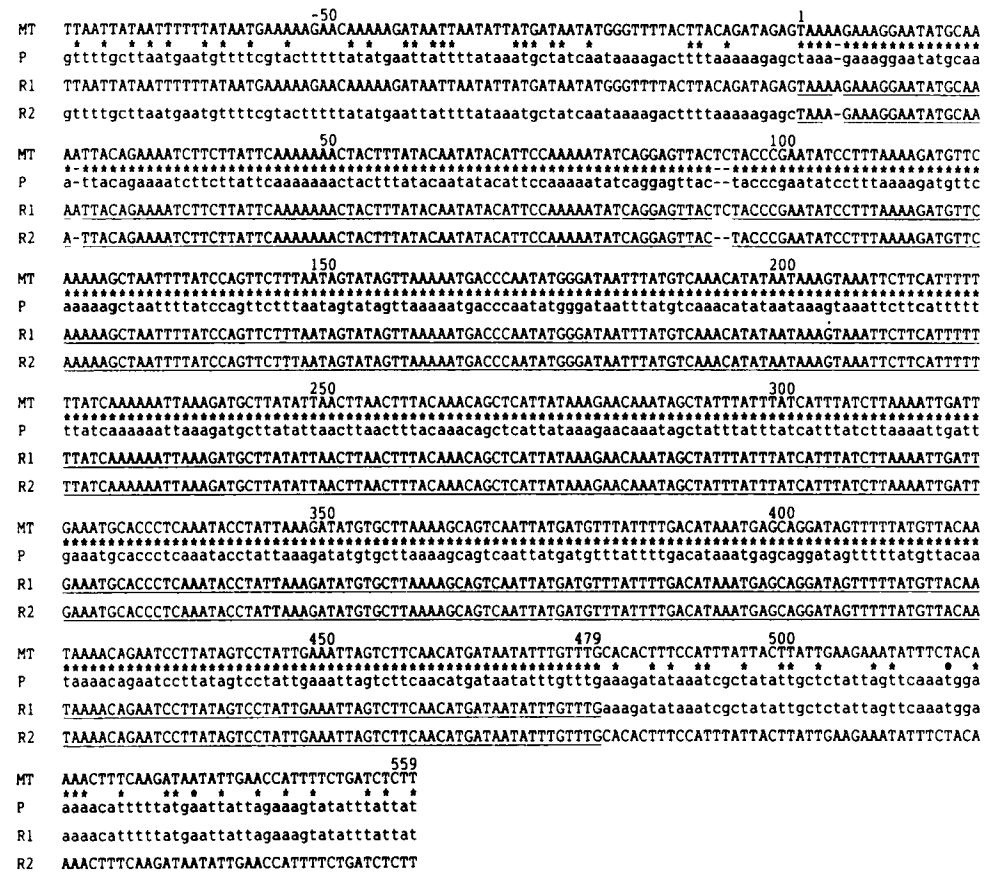
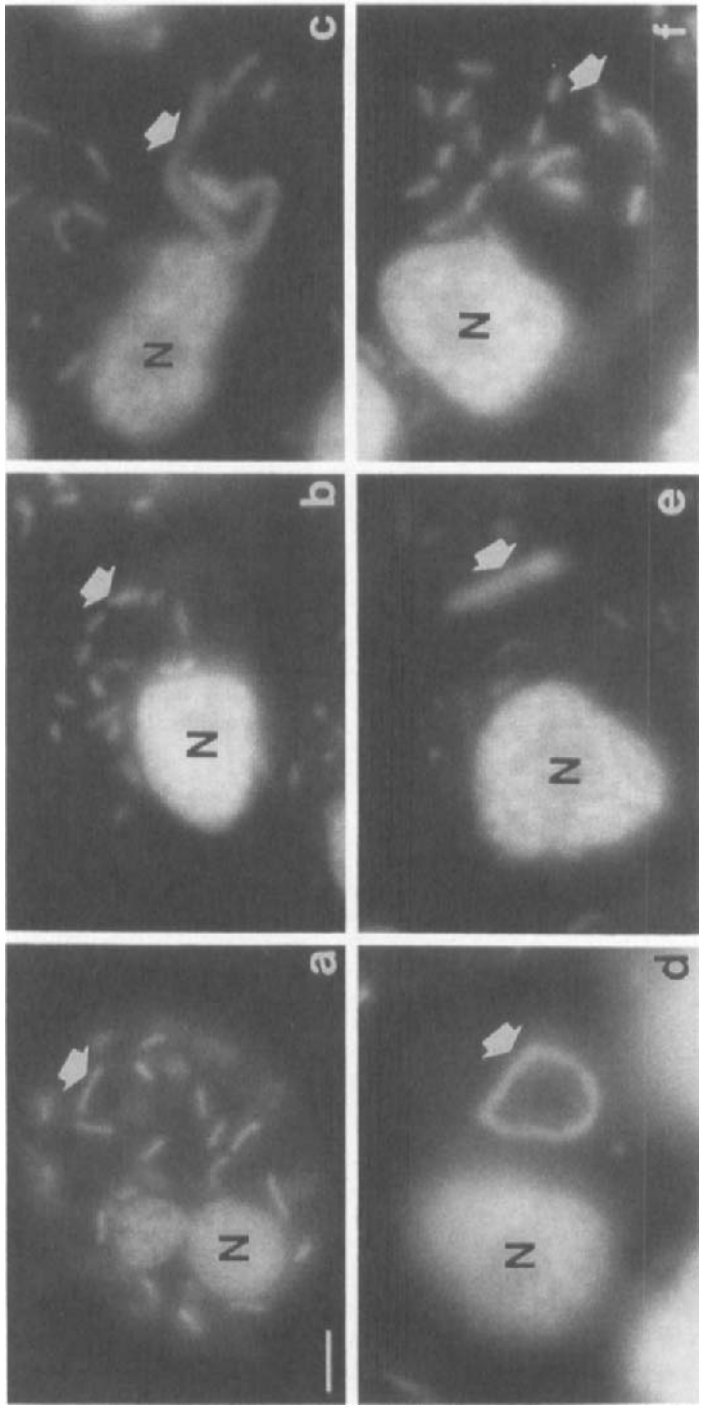


FIG. 12 Comparison of sequences between mtDNA (MT), mF plasmid (P), and recombinant mtDNA (R1 and R2). Sequences are numbered relative to the first nucleotide of the so-called identical sequence of the mtDNA, and matches between the mtDNA (capitals) and the mF plasmid (small letters) are indicated by asterisks. Sequences are shown from −80 to 559. Recombinant molecules are classified into two types: R1 consists of the minus region of mtDNA (−0), the so-called identical region (1–479) of mtDNA type, and the plus region of 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 (-). (From Takano et al., 1992.)

crossing-over at the left duplication, at the right duplication, and at both duplications. Each crossover event creates large and small recombinant mtDNAs. As shown in Fig. 12, one-half of the duplication of the mtDNA is recombined with the mF plasmid, and the small recombinant mtDNAs


a
b
c
d
e
f
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N

are derived from the left or right duplications. In each case, the end of the mF plasmid with extensive repeating units is located at the terminal of the recombinant mtDNA.

Integrations of linear plasmids have been also found in the mtDNAs of maize (Schardl *et al.*, 1985), *Claviceps purpurea* (Tudzynski and Esser, 1986), and *Neurospora* (Myers *et al.*, 1989; Court *et al.*, 1991). Among these plasmids, *kalilo* of *N. intermedia* has been well studied. It induces senescence in *Neurospora* by integration into the mtDNA (Bertrand *et al.*, 1985, 1986). The integration of *kalilo* occurs at seven distinct regions of the mtDNA and always generates very long inverted repeats of mtDNA flanking the two ends of the *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 is not flanked by inverted repeats of the mtDNA, and the recombination between the mtDNA and the mF plasmid is 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, S1 and S2 plasmids have been well studied. The same sequences in the mtDNA of maize, which are identical to those in the S1 and S2 plasmids, are derived from R1 and R2 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). It is possible that the R1 and R2 plasmids presumably integrated into the mtDNA and became fixed in a sequence identical to the S1 and S2 plasmids after deletion of other sequences. This may occur with the accompanying loss of the free plasmids. From such speculation, in the mitochondria of *P. polycephalum,* the mF plasmid is thought to be recombined with the mtDNA in an ancient strain and fixed as the so-called identical sequence after deletion of the entire sequence apart from the identical sequence and the free plasmid. This hypothesis is supported by the fact that the identical

FIG. 13 Mt-nuclear fusion and division in the zygote formation of *Physarum polycephalum.* The zygotes which were developing to plasmodia in the mating culture of mF^- x mF^+ were stained with DAPI. The rod-shaped mt-nucleus in individual mitochondria is observed in the binucleate zygote formed by myxamebal fusion (a). After cell-nuclear fusion, the mt-nuclei are queued (b) and fused to become a line (c) accompanying mitochondrial fusion. The fused mt-nuclei occasionally become circular (d) and tightly fused to become a large, rod-shaped mt-nucleus (e). Then, before the first plasmodial mitosis, the fused mt-nucleus successively divides to return to its former state (f). Arrows show mt-nuclei. N, nucleus. Bar = 1 μm.

sequence of the mF plasmid hybridized with the mtDNA of all strains during Southern hybridization (see Fig. 6).

2. Recombination and mt-Nuclear Fusion in Zygotes

It is obvious that the constitutive homologous recombination between mF plasmid and mtDNA occurs during plasmodium formation by a cross between mF^- and mF^+myxamebal strains. This suggests that the mitochondrial fusion accompanying mt-nuclear fusion occurs in the zygotes produced by such a cross (Fig. 13). Thus, these results suggest a picture of mitochondrial fusion events promoted by the plasmid, as illustrated in Fig. 14. In a zygote formed as a result of an $mF^+ \times mF^-$ mating, uninucleate mitochondria of two parental types fuse to form multinucleate mitochondria, in which the two main species of mtDNA and the plasmid are all present (Fig. 14A). After fusion of mitochondria, crossing-over occurs between homologous regions on the two main species of mtDNA or the plasmid. Recombinant mtDNA molecules and the unchanged free plasmid are then transmitted

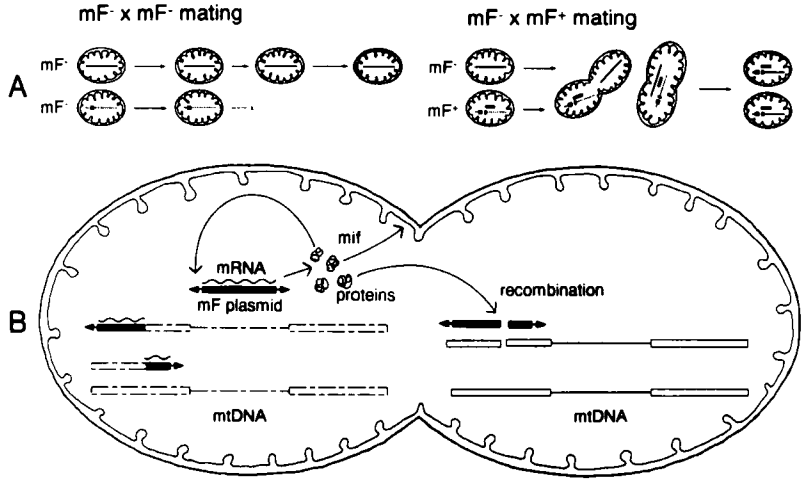

FIG. 14 Two transmission patterns of mitochondria (A) and events produced by the mF plasmid in a fused mitochondrion (B). In mF^- x mF^-, the mitochondria do not fuse but are transmitted uniparentally. In mF^- x mF^+, the mitochondria fuse and their mtDNA is recombined with the mF plasmid. The mitochondrial fusion is caused by the mF plasmid. The mF plasmid has three TIRs and an identical sequence with mtDNA, and codes 10 ORFs. The mF plasmid may be transcribed polycistronically from a specific site. The translated proteins of the mF plasmid may act on the mitochondrial fusion and recombination between the mtDNA and the mF plasmid in a fused mitochondrion.

to daughter mitochondria. A similar cycle also occurs during sporulation and the germination of spores (Fig. 1).

The transmission of the mF plasmid and the recombinant mtDNA in the mating between mF^- and mF^+ can be explained as follows (Fig. 14B). The mitochondria of two parental myxamebal 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 mF^- and mF^+, the recombinant mtDNA from the cross-over between the mtDNA of mF^- and the mF plasmid is transmitted to the plasmodium. It seems that the recombinant mtDNA is transmitted according to the *matA* hierarchy (*matA2* > *matA12*) with respect to the transmission of the mtDNA (Kawano and Kuroiwa, 1989; Meland *et al.*, 1991). The data in Fig. 12 suggest that the mtDNA of mF^+ (NG7) crosses over with the mF plasmid. Thus, mtDNA from mF^- or from mF^+ is transmitted to the plasmodium, and the sequence of the mF plasmid is maintained in the next generation as part of the recombinant mtDNA. Moreover, a free plasmid that does not cross 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 spread 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.

VI. Sexuality of Mitochondria

A. Evolution of Sex

A very obvious biological consequence of sex is the generation of new combinations of genes by mixing genomes, or portions thereof, from different individuals. This "mixis" has fundamental implications for both genetics and the evolutionary process. While we may agree that mixis is a fundamental consequence of sex, there is much less agreement concerning the mechanisms responsible for the origin and maintenance of sex. Heritable variation in fitness is the fuel of adaptive evolution, and sex can, although perhaps rarely, generate new combinations of alleles that are adaptive. However, it is not at all obvious whether the new adaptive combinations of alleles produced by sex are sufficient to confer a selective advantage on sexual individuals. There seems to be so far no consensus concerning the answer to the question of the adaptive significance of sex (Maynard-Smith, 1978; Ghiselin, 1988; Hamilton *et al.*, 1990).

In contrast, Hickey and Rose (1988) have taken a different approach altogether, challenging whether sex is advantageous to the individuals practicing it, and developed a multistage scenario for the evolution of sex in which parasitic gene transfer is the dominant factor. In this schema, sex could be the result of the evolution of parasitic selfish DNA molecules that exploit the opportunities for horizontal transmission afforded by cycles of germ cell fusion and fission to spread horizontally through populations. The parasitic selfish DNA molecules could, in theory, enhance their own fitness by promoting gamete fusion (Hickey, 1982, 1984). The formal analyses in which both the origin and the maintenance of such "parasitic sex" have been considered to be of central importance (Rose, 1983; Tremblay and Rose, 1985; Krieber and Rose, 1986) suggest that such parasitic gene transfer could indeed play an important role in the evolution of eukaryotic sex, although adaptive benefits to the host cells might also play a critical role. A similar argument for the role of transmissible genes in evolution of bacterial sex has been outlined by Zinder (1985).

The important conclusion of the "parasitic origin of sex" theory is that transposable elements that have positive, neutral, or negative effects on host fitness can all spread within a sexual population in a way that would not be possible within an asexual population. Thus this advantage of sexuality for the transposable elements implicates them in the origin of sex. A coincidental evolution hypothesis for mixis is also favored for plasmid- and phage-mediated recombination (Hickey and Rose, 1988). In these cases, sex, the capacity for infectious transfer, is encoded by genes borne by the plasmid or phage, rather than by host genes, and the mixis of host genes is viewed as a coincidental by-product of evolution of the plasmid or phage. Such parasite coevolution seems to be superior to previous models of the evolution of sex by supporting the stability of sex under the following challenging conditions: very low fecundity, realistic patterns of genotype fitness and changing environment, and frequent mutation to parthenogenesis, even while sex pays the full two-fold cost (Hamilton *et al.*, 1990; Howard and Lively, 1994). Both the theoretical calculations and the experimental observations support the idea that self-replicating, intragenomic elements can gain a large advantage from the sexual mixing of their host genomes.

B. Bacterial Sex

The best clues as to the probable nature of primitive sex come from the study of living prokaryotes rather than eukaryotes (Jacob and Wollman, 1961; Margulis and Sagan, 1986; Levin, 1988). In eukaryotes, a single specialized sexual system based on cycles of syngamy and meiosis is virtually ubiquitous. In contrast, the prokaryotes still display a wide variety of sexual

and asexual reproductive systems. The prokaryotic sexual systems are both conjugative and nonconjugative. The genes of prokaryotes are infected by one of three basic mechanisms—transformation, transduction, and conjugation. With transformation, free DNA is taken up by the bacterium and incorporated into its genome. In transduction, the movement of genetic material between cells is accomplished by bacteriophage vectors that pick up DNA from a donor cell and transmit it to a recipient. Conjugation is a more intimate process, with the transfer of genes requiring contact between a donor and a recipient cell.

The first genetic evidence for sex via conjugation was obtained in *Escherichia coli* (Lederberg and Tatum, 1946). The observation of conjugation between bacterial cells was taken as direct evidence for sexual mating in this species. This process is genetically controlled by a self-transmissible conjugative plasmid, the F factor (Ippen-Ihler and Minkley, 1986; Smith, 1991). More than 60 genes and sites have been located on the physical and genetic map of the F plasmid, a covalently closed, circular, double-stranded DNA molecule close to 100 kbp in length. The capacity of *E. coli* to transfer chromosomal genes by conjugation and the variety of changes in the donor cell surface associated with the transfer process are determined by genes carried by the F plasmid. The integration of the plasmid and chromosome, forming a state known as a "Hfr" (high-frequency recombination), can occur in a number of places along the circular *E. coli* chromosome. Transfer is polar, originating with a specific sequence on F, not surprisingly known as the origin of transfer (*oriT*), and immediately followed by the regions of the F plasmid associated with replication. The genes responsible for the self-transmissibility of the plasmid enter the recipient last. In Hfr, the entire bacterial chromosome (about 50 times the length of F) separates the origin of transfer from the genes required for conjugation.

The idea of a relationship between bacterial parasites and a form of bacterial sex is not a new one. These bacteriophages, which act as generalized transducing phages, are potentially important in recombination yet they are clearly bacterial parasites. Dougherty (1955) was one of the first to point out that phages, although they are parasites, could also facilitate a form of bacterial sexuality. The essence of the "parasitic origin of sex" theory is not only that intracellular parasites provide a form of sexuality among prokaryotes, but also that "conventional" eukaryotic sex itself began with a type of intracellular parasitism. As regarded with the evolution of sex as a multisite process, where each step evolved as a function of the individual selection of genes that were favored under conditions existing at a particular stage. This is not surprising if primitive eukaryotic mating, or syngamy, was the first step in a complex evolutionary process starting from such conjugation. During the course of the ensuing process, syngamy has taken on a new significance.

C. Mitochondrial Sex

As outlined in the preceding section, both mathematical and biological arguments suggest that there is no appreciable problem with hypothesizing an initially parasitic origin of sex. It is highly unlikely, however, that sex has since continued to be simply parasitic. No biological system that displays the initial stages in a parasitic origin of sex has been described. According to the endosymbiosis theory, mitochondria originated as free-living cells that were later the prey, or parasites, of the ancestors of modern eukaryotic cells. Proponents of the endosymbiotic theory would not, however, consider modern mitochondria as an illustration of the initial parasitic origin of sex; although they have obviously become an integral part of the eukaryotic cell, they are highly infectious, with mobile genetic elements such as ω-like introns and plasmids and have the ability to fuse and recombine. Mitochondria and chloroplasts have their own genomes, which are different from those of the cell nuclei. The origins of these organelles have been regarded as a line in the evolution of the aerobic bacteria or green algae that cohabited in primordial cells during the establishment of eukaryotic cells. A possibility remains that the primordial sex of the symbionts was inherited as an attribute of the organelles. If so, the recombination mediated by parasitic selfish DNA such as conjugative plasmids must take place between different organelles. This is very similar to that theory proposed by Hickey and Rose (1988) for the initial stage in the evolution of parasitic sex. The mF plasmid that promotes mitochondrial fusion in *Physarum polycephalum* provides direct support for the possibility that selfish genes are capable of manipulating their hosts and developing sexuality as pointed out by Hurst (1991). This is very similar to bacterial conjugative plasmids such as the F plasmid in *E. coli* (Fig. 15). Such similarity indicates the origin not only of themselves but also of their sex.

Recombination between mitochondrial genomes has also been found in a few other organisms, the best studied of which is the yeast *Saccharomyces cerevisiae.* This recombination can involve biased gene conversion, or a similar process at the ω locus such that in a cross between cells which are ω^+ and ω^-, nearly all of the mitochondria end up being ω^+. Unlike the case of *P. polycephalum,* ω^+ is not a plasmid but an intron. It is not known if ω^+ can induce mitochondrial fusion, but its infectious feature is well known (Dujon, 1989; Lambowitz and Belfort, 1993). Moreover, in *S. cerevisiae,* recombination of mtDNA is known to take place between mitochondria in the absence of ω^+. In contrast, the mF plasmid promotes mitochondrial fusion, but the recombination of mtDNA without the mF plasmid is not known in *P. polycephalum.* There are very serious differences in the manner of mitochondrial fusion and recombination in these two characteristic organisms. Such differences likely reflect the multistage scenario for the evolution of sex in which parasitic gene transfer is the dominant factor. The

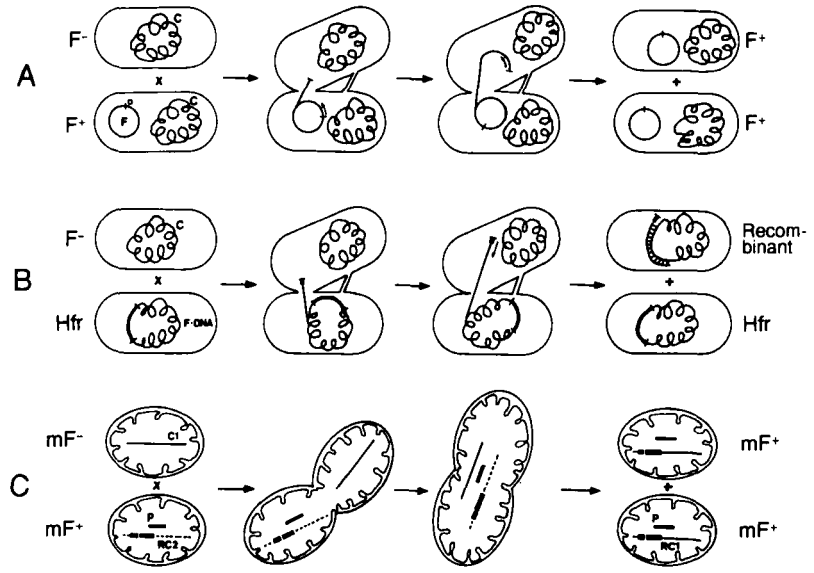

FIG. 15 Bacterial sex and mitochondrial sex: conjugation and gene transfer mediated by the F plasmid of *Escherichia coli* (A, B), and mitochondrial fusion and recombination mediated by the mF plasmid of *Physarum polycephalum* (C). Heavy lines represent plasmid genomes; light lines, the host chromosomes. The origin of conjugative DNA transfer is indicated by a vertical short line. Donor and recipient bacterial cells are shown in the process of conjugation, joined by the pilus. (A) F plasmid conjugation. Only one copy of the F plasmid is transferred. (B) Hfr-mediated host gene transfer. The F plasmid of the donor is integrated into the host chromosome. During conjugation, a copy of a portion of the donor chromosome is transferred to the recipient, where it may replace parts of the recipient chromosome by homologous recombination. (C) mF plasmid-mediated mitochondrial fusion. The thin and dotted lines represent the mtDNA in individual mitochondria of two parents. Following mitochondrial fusion and mt-nuclear fusion, crossing-over occurs between homologous regions on the mtDNA and the mF plasmid. Newly formed recombinant mtDNA and unchanged mF plasmid are then transmitted to daughter mitochondria preferentially.

mitochondria are very much infectious; numerous mobile genetic elements are known in the mitochondria of some organisms, as described in Section III. Such an infectious situation in mitochondria also suggests the existence of other types of primordial sex mediated by some mobile genetic element and provides a useful tool for analyzing the parasitic origin of sex.

VII. Concluding Remarks

In this chapter, mitochondrial fusion, recombination, and mobile genetic elements, which are essential for mitochondrial sexuality, are outlined. These

phenomena seem to be ubiquitous. Nevertheless, their roles in mitochondrial biogenesis and sexuality remain somewhat obscure, as do the molecular mechanisms that control mitochondrial fusion in most organisms, with the notable exception of *Saccharomyces cerevisiae* and *Physarum polycephalum.* The existence of the mF plasmid that promotes mitochondrial fusion and recombination in *P. polycephalum* should provide direct evidence for primordial sex in mitochondria and mitochondria as biological entities in the initial stages of the evolution of parasitic sex. The processes of mt-nuclear fusion, recombination, and segregation, which are demonstrated in *P. polycephalum,* provide evidence of a kind of mitochondrial "meiotic" cycle. Cell-nuclear meiosis is a process closely related to the sexuality of eukaryotes; primitive sexuality and recombination of chromosomes are even observed in bacteria. The possibility that mitochondria possess a primitive mode of sexuality similar to that of bacteria does not conflict with the postulate that mitochondria originated as the result of bacterial endosymbiosis. Making the best use of these favorable features of *P. polycephalum* and advancing studies on the mitochondrial fusion and recombination in some other organisms, it should be possible to establish the phenomenon which we have termed "mitochondrial sex" in the field of mitochondrial biogenesis.

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