Polymorphism and Uniparental Inheritance of Mitochondrial DNA in *Physarum polycephalum*

By S. KAWANO,¹* R. W. ANDERSON,² T. NANBA¹ AND T. KUROIWA¹

¹ Department of Cell Biology, National Institute for Basic Biology, 38 Myodaijicho, Okazaki 444, Japan

² Department of Genetics, University of Sheffield, Sheffield S10 2TN, UK

(Received 10 April 1987; revised 22 June 1987)

Restriction endonuclease analysis was done on mitochondrial DNA (mtDNA) from 19 plasmodial strains of *Physarum polycephalum*. The extent of mtDNA variation among these strains was high in comparison with other organisms, and provides a useful source of cytoplasmic genetic markers. The strains were classified into seven groups according to their mtDNA types. Although plasmodia of *P. polycephalum* are diploid, formed by fusion of amoebal isogametes, each of the 19 plasmodia possessed mtDNA of only a single type. The transmission pattern of mtDNA during plasmodium formation was studied by mating pairs of amoebal strains that contained mtDNA of different types. Transmission was 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 mitochondria or of mtDNA molecules during cell division.

INTRODUCTION

Mitochondrial genes are transmitted to progeny predominantly or entirely by only one parent in a number of different organisms. In oogamous species, this uniparental inheritance has usually been attributed to failure of mitochondria from the male gamete to enter the egg, or to the presence in the male gamete of comparatively few organelles (for reviews see Sager, 1972; Birky, 1978). However, these cellular mechanisms cannot explain the production of uniparental, homoplasmic zygotes in species such as yeasts, which do not have differentiated gametes; in such isogamous species, uniparental inheritance is thought to be due at least partly to random partitioning of mitochondria during cell division (for reviews see Birky, 1978; Birky *et al.*, 1982).

The life cycle of the acellular slime mould *Physarum polycephalum* includes two distinct vegetative forms: the uninucleate amoeba and the multinucleate, syncytial plasmodium. Haploid amoebae act as isogametes, fusing in pairs of different mating type to form diploid zygotes which develop into macroscopic, diploid plasmodia by repeated mitotic cycles in the absence of cell division (reviewed by Dee, 1982). Thus, the random partitioning of mitochondria by cell division does not occur during plasmodium formation. It was of interest to determine whether a heteroplasmic zygote produced by mating would develop into a homoplasmic plasmodium in spite of the absence of such random partitioning.

The inheritance of mitochondrial genes in *Physarum* has been difficult to study because easily scored cytoplasmic mutants have not been available. In the present work, restriction endonuclease digestion has been used both to study the degree of mtDNA polymorphism among a series of *P. polycephalum* strains, and to determine the pattern of transmission of mtDNA during the process of plasmodium formation.

Abbreviation: mtDNA, mitochondrial DNA.

Table 1. *Strains used*(a) *Plasmodial strains*

Designation	Reference or origin
1 RA669 × RA670	
2 M ₃ b	Mohberg & Babcock (1982)
3 M ₃ c	Mohberg & Babcock (1982)
4 At	ATCC 44912
5 LU897 × LU898	
6 CH929 × CH938	
7 OX115 × RA263	
8 Os	N. Kamiya, Osaka University
9 F × J	Wakasugi & Ohta (1973)
10 PPO-1	Kawano <i>et al.</i> (1982)
11 Cb	F. Sawada, National Institute for Radiological Sciences
12 To	A. Nishi, Toyama Medical & Pharmacological University
13 Ho	Y. Kobatake, Hokkaido University
14 DP74 × DP75	
15 Nc	North Carolina biological supply house
16 Ng	S. Hatano, Nagoya University
17 Hi	E. Kamitsubo, Hitotsubashi University
18 DP12 × DP13	
19 DP89 × DP90	

(b) *Amoebal strains*

Designation	Genotype	Reference or origin
a	<i>matA1 matB1</i>	Dee (1960)
i	<i>matA2 matB4</i>	Dee (1960)
CH929	<i>matA1 matB3</i>	Anderson & Truitt (1983)
CH938	<i>matA4 matB2</i>	Kawano <i>et al.</i> (1987)
DP12	<i>matA5 matB10</i>	Kirouac-Brunet <i>et al.</i> (1981)
DP13	<i>matA6 matB11</i>	
DP74	<i>matA12 matB5</i>	
DP75	<i>matA11 matB6</i>	
DP89	<i>matA15 matB12</i>	
DP90	<i>matA16 matB13</i>	
LU853	<i>matA1 matB1</i>	Cooke (1974)
LU887	<i>matA2 matB1</i>	Anderson (1977)
LU897	<i>matA1 matB1</i>	Anderson (1976)
LU898	<i>matA2 matB1</i>	LU853 × LU887
OX110	<i>matA2 matB1</i>	Kawano <i>et al.</i> (1987)
OX115	<i>matA3 matB1</i>	
OX118	<i>matA3 matB3</i>	
RA263	<i>matA2 matB3</i>	OX110 × OX118
RA669	<i>matA1 matB4</i>	a × i
RA670	<i>matA2 matB1</i>	a × i

METHODS

Strains. Principal plasmodial and amoebal strains are listed in Table 1. Amoebae were maintained at 29.5 °C in monoxenic culture with *Escherichia coli* on SM-1 plates, as described by Kawano *et al.* (1987). Plasmodia were cultured at 26 °C on MEA (malt extract agar; Kawano *et al.*, 1987) and microplasmodia were cultured as described by Daniel & Baldwin (1964). Sporulation was induced on MEA plates, essentially as detailed by Wheals (1970). Germination of spores and cloning of amoebal progeny were done according to the methods of Anderson (1979) but using SM-1 instead of liver infusion agar. Stocks of amoebal strains were maintained as desiccated cysts on silica gel granules (Anderson *et al.*, 1983).

Generation of plasmodia and mating-type analysis. To obtain plasmodia by mating, 10⁵ amoebae of each of two strains carrying unlike *matA* and *matB* alleles were mixed at 26 °C in 'concentrated' live *E. coli* suspension and plated as described by Youngman *et al.* (1981). Following mating, small agar blocks carrying plasmodia were cut from the mating plates and transferred to MEA for further growth. These plasmodia were confirmed to result from mating by analysis of their progeny for *matA* and *matB* alleles. Such analyses were done essentially as described by Youngman *et al.* (1979).

Table 2. Classification of plasmodia for mtDNA types and mating-type alleles

Strain	Nucleus Mating type	Mitochondria			mtDNA type
		Genome size	Percentage differential restriction fragments		
			(a)*	(b)†	
RA669 × RA670, M ₃ b, M ₃ c, At, LU897 × LU898, CH929 × CH938, OX115 × RA263	<i>matA1-4</i> <i>matB1-4</i>	60.1 ± 4.3	—	25.9	M (McArdle)
Os, F × J, PPO-1, Cb, To, Ho, DP12 × DP13	Unknown‡ <i>matA5,6</i> <i>matB10,11</i>	68.2 ± 8.8	43.2	44.9	O (Osaka)
DP89 × DP90	<i>matA15,16</i> <i>matB12,13</i>	64.7 ± 3.8	37.6	39.2	I (Iowa)
DP74 × DP75, Nc	<i>matA11,12</i> <i>matB5,6</i>	61.1 ± 7.2	18.8	15.9	W (Wisconsin)
Ng	<i>matA11,12</i> <i>matB5,6</i>	62.1 ± 3.6	25.9	—	B (Boreal)
Hi	<i>matA11,12</i> <i>matB5,6</i>	62.7 ± 5.7	27.0	17.1	N (Nagoya)
		63.4 ± 4.5	30.7	16.1	H (Hitotsubashi)

* Comparison with M-type.

† Comparison with B-type.

‡ Not *matA1,2,3,4,5,6,11,12,15,16* or *matB1,2,3,4,5,6,10,11,12,13*.

Restriction endonuclease analysis of mtDNA. Mitochondria were isolated from microplasmodia or macroplasmodia of each strain as described by Kawano *et al.* (1983). mtDNA was isolated from isolated mitochondria by centrifugation in Hoechst 33258/CsCl density gradients according to Hudspeth *et al.* (1980). Restriction endonucleases were obtained from New England Biolabs and the Takara Shuzo Co. Digests were done in buffers recommended by the manufacturers. Electrophoresis and photography of 1–2% (w/v) agarose slab gels were as described by Kawano *et al.* (1983). Percentage differential restriction fragments (p_{ij}) were calculated by the formula $p_{ij} = 100(d_{ij}/n_i + n_j)$, where n_i and n_j are the total fragment numbers observed in the restriction patterns of the i th and j th mtDNA types and d_{ij} is the number of differential fragments found between the two types as described by Kaneko *et al.* (1986). This analysis was done by using the data of six-base-pair-recognizing restriction endonucleases (*EcoRI*, *EcoRV*, *HindIII*, *HpaI* and *XbaI*).

RESULTS AND DISCUSSION

Polymorphism of mtDNA revealed by restriction endonuclease analysis

mtDNA isolated from microplasmodia of 19 strains was digested with six restriction endonucleases: *Bst*NI, *EcoRI*, *EcoRV*, *HindIII*, *HpaI* and *XbaI*. Restriction fragment patterns sometimes deviated from normal stoichiometry; in particular, restriction fragments from the terminal regions of mtDNA molecules tended to be seen as faint and diffuse bands (Fig. 1). As reported by Kawano *et al.* (1982), this phenomenon is attributable to the use of broken linear mtDNA as starting material. Partial breakage of mtDNA is generally observed during isolation of mtDNA from fungi (Sanders *et al.*, 1975) and does not prevent the estimation of mitochondrial genome size or the detection of mtDNA variation. The mitochondrial genome size estimated from the total molecular masses of the restriction fragments differed among the strains (60.1–68.2 kb; see Table 2), but the values were all close to the genome size of 68.2 kb found for strain PPO-1 by Kawano *et al.* (1982). As shown in this earlier study, this implies that the mtDNA within each individual strain was of only a single type.

Fig. 1(a) shows the *HindIII* restriction fragment patterns of mtDNA from the 19 strains.

Fig. 1. (a) *Hind*III restriction patterns of mtDNA from the 19 plasmodial strains. The lanes are numbered in accordance with Table 1 (a). (b)–(d), Restriction patterns of the seven mtDNA types with *Bst*NI (b), *Eco*RV (c), *Xba*I (d). The lanes are numbered as follows: 1, M-type mtDNA; 2, O-type; 3, B-type; 4, N-type; 5, H-type; 6, I-type; 7, W-type.

Seven distinct types of mtDNA were detected. Fig. 1(b–d) shows representative restriction fragment patterns of these seven distinct mtDNA types digested with *Bst*NI, *Eco*RV and *Xba*I. The digestion patterns showed conservation of certain restriction fragments between the different mtDNA types, but most fragments were unique to each type. Digestions with *Eco*RI and *Hpa*I (data not shown) also confirmed the existence of seven groups. The detailed pattern of methylation sites in *P. polycephalum* mtDNA is unknown, although the involvement of cytosine is clearly established (Kawano *et al.*, 1983); since *Bst*NI, *Eco*RI, *Eco*RV and *Hpa*I are not affected by methylation of cytosines, it is clear that the different restriction patterns were largely based upon differences in nucleotide sequence rather than on differential methylation.

To quantify simply the variation between different mtDNA types, percentage differential restriction fragments, p_{ij} , were calculated; these show the frequency of differential restriction fragments observed in the pairwise comparison of the different mtDNA types (Table 2). The mean value of p_{ij} was 28.5% (SD = 10.2%), significantly higher than the percentage differential restriction fragments of animal mtDNA, which range from 2.4% in humans to 7.8% in chimpanzees. These values were calculated from the data of Cann *et al.* (1984) and Ferris *et al.* (1981), respectively. Strain and specific variations in mtDNAs have also been observed in many fungi, and variation here is generally greater than among animal mtDNAs. For example, in *Achlya heterosexalis* (Shumard *et al.*, 1986), the percentage differential restriction fragments were calculated to be 18.0%.

It is known that polymorphism of animal mtDNA mostly results from single base substitutions (Castora *et al.*, 1980), but it has been postulated that variation in fungi is the result of insertions into or deletions from basically similar genomes in which most restriction endonuclease sites are conserved (Turner *et al.*, 1982). The mitochondrial genome size heterogeneity observed among *P. polycephalum* strains suggests that variations of restriction fragment patterns may in part be caused by such insertions and deletions. Nevertheless, the basis of genome size heterogeneity and nucleotide diversity in *P. polycephalum* mtDNA is not yet

certain. The high degree of variability defined in these studies makes it clear that polymorphism of mtDNA is a potentially rich source of cytoplasmic genetic markers in this organism, which has previously lacked any well-defined cytoplasmic mutants.

Origins of the different mtDNA types

The existence of seven distinct types of mtDNA among the 19 strains studied suggested that each mtDNA type might correspond to a different natural plasmodial isolate of *P. polycephalum*. Some support for this view came from the known common origins of certain strains; in many cases, however, the original isolate was unknown, and information was therefore obtained from tests of mating type. Each natural plasmodial isolate of *P. polycephalum* appears to be characterized by two unique alleles of each of the multiallelic mating-type loci *matA* and *matB* (Kirouac-Brunet *et al.*, 1981), so that mating-type alleles are good nuclear gene markers to show the particular isolate from which any strain has been derived.

M₃b and M₃c are sublines of the same isolate, Wis 1, collected by M. Backus about 1947 (Mohberg & Babcock, 1982); RA669 and RA670 are second-generation meiotic progeny derived from the same isolate. M₃b, M₃c and RA669 × RA670 all carried mtDNA of the same type, designated M-type (Table 2). The plasmodium designated At also carried M-type mtDNA, and analysis of its amoebal progeny showed that the mating-type alleles characteristic of the Wis 1 isolate were present: *matA1*, *matA2*, *matB1* and *matB4*. It thus appears that the At plasmodium, which was obtained from the American Type Culture Collection, was of Wis 1 origin.

Three further plasmodial strains carried M-type mtDNA. These plasmodia were all generated by crosses between pairs of amoebal strains that were largely isogenic with the Colonia isolate, although the strains carried mating-type alleles derived from the Wis 1 and Indiana isolates (Adler & Holt, 1974; Cooke & Dee, 1975). The Colonia and Wis 1 isolates are genetically similar in several ways and it has been suggested that Colonia is probably derived from Wis 1 (Honey *et al.*, 1979; see also Kawano *et al.*, 1987). The fact that Colonia-isogenic strains possess M-type mtDNA is consistent with this hypothesis.

The six plasmodial strains possessing O-type mtDNA were all obtained from Japanese laboratories. These plasmodia carried identical *matA* and *matB* alleles, which were different from those carried by any of the other plasmodia or by any of the mating-type tester strains available to us: *matA1*, 2, 3, 4, 5, 6, 11, 12, 15, 16, and *matB1*, 2, 3, 4, 5, 6, 10, 11, 12, 13.

The I and W mtDNA types were each represented by a single plasmodium with unique mating-type alleles. DP12 × DP13 was derived from progeny of the plasmodial isolate PpII (Collins, 1975), and DP89 × DP90 was derived from progeny of the Wis 2 isolate (Kirouac-Brunet *et al.*, 1981).

Two plasmodia possessed B-type mtDNA: Nc was obtained from the North Carolina biological supply house, and DP74 × DP75 was generated by mating amoebal progeny of a plasmodium from the Boreal biological supply house. On the basis of mating-type analyses, Kirouac-Brunet *et al.* (1981) suggested that the Boreal, North Carolina and Turtox supply houses were all culturing the same plasmodium, originally known as the Turtox isolate. Our own mating-type analyses confirm that Nc carries the same alleles as DP74 × DP75: *matA11* and *matA12*, *matB5* and *matB6*.

Although Ng and Hi each carried a unique mtDNA type, mating-type analyses showed that both these plasmodia carried the same *matA* and *matB* alleles as Nc and DP74 × DP75. Calculation of percentage differential restriction fragments showed that the three mtDNA types involved (B, N and H) were not closely related, and thus did not represent minor divergence from an ancestral sequence in the original wild isolate. Unless that isolate carried three different mtDNA species which have since become segregated into different sublines, it would appear that exchanges of cytoplasm must have occurred between isolates. However, it is difficult to see how such exchanges would take place. Mating between amoebae of different isolates occurs readily, but the plasmodial products of such matings contain only a single *matA* and *matB* allele from each parent. A second possible mechanism of cytoplasmic exchange would be plasmodial fusion, but the large number of incompatibility genes controlling somatic fusions between

Fig. 2. Uniparental transmission pattern (lanes 3–6) of mtDNA during plasmodium formation by the mating between amoebal strains MB29 (lane 1) and NG9 (lane 2). MtDNAs were digested with *Bst*NI (a) or *Xba*I (b). For each of the four plasmodia, the mtDNA digestion patterns were of M-type, the same as MB29.

plasmodia makes it unlikely that plasmodia of different isolates would be able to fuse (Collins, 1972; Collins & Haskins, 1972).

Uniparental inheritance of mtDNA during plasmodium formation

The restriction analyses of mtDNA showed that all the plasmodial strains were homoplasmic, i.e. possessing only one type of mtDNA. This suggested that uniparental transmission of mtDNA might occur during plasmodium formation, and the possibility was investigated by mating amoebal strains that carried mtDNA of different types. Amoebal strains MB29 (*matA2 matB1*; M-type) and NG9 (*matA11 matB6*; N-type) were isolated as haploid, meiotic progeny of the plasmodial strains M₃b and Ng, respectively. Plasmodia were obtained from four independent matings of these amoebal strains and mtDNA from each of the plasmodia was isolated and analysed by restriction endonuclease digestion (Fig. 2). Each plasmodium possessed only one parental type of mtDNA, and in every case it was the M-type. This result demonstrated uniparental inheritance of mtDNA, and suggested a possible bias in favour of one parent (MB29).

Amoebae of *P. polycephalum* are isogametes. It was confirmed by microscopy (unpublished data) that amoebae of strains MB29 and NG9 were approximately equal in size and in content of mitochondria. Thus, it would be expected that amoebae of these strains should contribute roughly equal numbers of mitochondria to heteroplasmic zygotes at the time of fusion. Zygotes develop into multinucleate, syncytial plasmodia by repeated mitotic cycles in the absence of cell division, as well as by fusions among developing plasmodia. Even at the earliest time at which analysis of plasmodia could be done, some 30–40 mitotic cycles after amoebal fusion, there was no evidence that more than one mtDNA type was present. It thus appears that the production of homoplasmic cells from heteroplasmic ones must occur relatively early in the process of vegetative growth and fusion.

A well-documented feature of yeast mitochondrial genetics (reviewed by Birky, 1978; Birky *et al.*, 1982) is that heteroplasmic cells may produce homoplasmic progeny, so that alleles appear to segregate during mitotic as well as meiotic divisions. This vegetative segregation is caused in part by random partitioning of cytoplasmic organelles or DNA molecules between daughter cells at each cell division. Another factor is intracellular drift of gene frequencies, due largely to random sampling of organelle DNA molecules for replication. In plasmodium formation by *P.*

polycephalum, however, random partitioning of mitochondria or of mtDNA molecules by cell division cannot be a significant factor in vegetative segregation. The biased uniparental transmission of mtDNA suggests that a non-random mechanism may be operating, such as preferential replication of one of the two mtDNA species. To determine the nature of such non-random processes, it will be necessary to develop methods to study the precise behaviour of mitochondria and mtDNA molecules during the earliest stages of plasmodial development.

The uniparental inheritance of mtDNA documented in the present work is only one of several phenomena involving vegetative segregations in *P. polycephalum*. For example, plasmodial heterokaryons containing both haploid and diploid nuclei show instability, breaking down to yield homokaryons containing nuclei only of the original haploid component (Dee & Anderson, 1984); it is thought that this type of instability may be involved in the phenomenon of 'stimulated selfing' (Anderson & Truitt, 1983), in which the plasmodia arising in a cross are sometimes found to include haploids of parental genotype as well as diploids. Another kind of heterokaryon instability is under the control of incompatibility genes known as *let* (Carlile, 1976) or *kil* (Adler & Holt, 1974). Following plasmodial fusion, nuclei carrying dominant *let* alleles apparently cause destruction of nuclei carrying recessive alleles of the same *let* loci (Border & Carlile, 1974; Lane & Carlile, 1979). It will be of interest to determine whether the mechanism of uniparental inheritance of mtDNA in any way parallels these examples of uniparental nuclear inheritance.

We greatly thank Drs M. J. Carlile, S. Hatano, E. Kamitsubo, N. Kamiya, Y. Kobatake, K. Kuroda (Osaka University), A. Nishi, J. Ohta (Ochanomizu University), Y. Sakaki (Kyushu University), F. Sawada and D. Pallotta (Laval University) for the gifts of strains. This work was supported in part by Grant no. 60740391 (S.K.) and a research scholarship abroad from the Ministry of Education, Science and Culture of Japan.

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