UNIDIRECTIONAL GENE CONVERSION ASSOCIATED WITH TWO INSERTIONS IN *NEUROSPORA CRASSA* MITOCHONDRIAL DNA

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ABSTRACT

The mitochondrial phenotype of [poky] and other extranuclear Neurospora mutants is known to predominate over that of wild type in heteroplasmons. In the present work, we have investigated the interaction between wild-type and [poky] mtDNAs using as many as four physical markers to distinguish the two types of mtDNAs. Two insertions, one of 1200 bp in Eco RI-5 and the other 50 bp in Eco RI-9, are identified as sites of high frequency, unidirectional gene conversion leading to their spread through mtDNA populations in heteroplasmons. However, the transmission of the [poky] mutation does not appear to be correlated with the transmission of either of these insertions or of other physical markers. The possibility that other loci of nonreciprocal recombination might be responsible for the "dominance" of Neurospora extranuclear mutants is discussed.

IN previous work, we made use of restriction-enzyme analysis to study the segregation of *Neurospora crassa* mtDNA in sexual crosses and in heteroplasmons (MANNELLA and LAMBOWITZ 1978; MANNELLA, PITTENGER and LAMBOWITZ 1979). These experiments showed that recombination between mtDNA molecules occurs frequently in heteroplasmons and that mixtures of mtDNA molecules present upon formation of the heteroplasmons are rapidly resolved, so that a single mtDNA species, either a parental or recombinant type, comes to predominate (MANNELLA and LAMBOWITZ 1978). In sexual crosses, mtDNA shows strict maternal inheritance with a lack of recombinant types that are frequently observed in heteroplasmons (MANNELLA, PITTENGER and LAMBOWITZ 1979).

One objective of our research has been to investigate the interaction between mtDNAs from wild-type and extra-nuclear mutants in heteroplasmons. Early studies by PITTENGER (1956) and DIACUMAKOS, GARNJOBST and TATUM (1965) showed that the mitochondrial phenotype of [poky] ([mi-1]) and other extranuclear mutants predominates over the wild-type phenotype in heteroplasmons formed by heterokaryosis or microinjection of mutant mitochondria. TATUM and LUCK (1966) suggested that the predominance of the mutant phenotype might reflect a replicative advantage of the mutant over the wild-type genetic determinant (presumably mtDNA). In previous work, we attempted to use our

experimental system to test the replicative advantage model, but the results were complicated by the high frequency of recombination between the parental mtDNAs (MANNELLA and LAMBOWITZ 1978).

In the present study, we have made use of wild-type ([+]) and [poky] parental strains containing mtDNAs with additional physical markers in an attempt to carry out a more detailed examination of the interaction between wild-type and [poky] mtDNA in heteroplasmons. Our results provide definitive evidence for recombination between mtDNA molecules in heteroplasmons. In addition, we identify two insertions as sites of high frequency, unidirectional gene conversion leading to their spread through mtDNA populations in heteroplasmons. The possibility that such loci play a role in the "dominance" of Neurospora extranuclear mutants is discussed.

MATERIALS AND METHODS

Neurospora strains: The strains of Neurospora used in the present study were constructed so as to have heterokaryon-compatibility alleles of wild-type strain 74-OR-23-1A (abbreviated 74A). The strains were used to construct three sets of heteroplasmons (H1, 2 and 3) containing different combinations of mtDNA types (summarized in Table 1). The H1 set consists of five heteroplasmons formed by mycelial fusion between [II, +] 237 *nic-1 al-2 a* and [IIa, poky] PU-5 *pan-2 a*. These heteroplasmons correspond to numbers 6 to 10 described previously (MANNELLA and LAMBOWITZ 1978), but with mtDNAs analyzed in greater detail. The H2 set consists of three heteroplasmons formed between [I, +] *pan-2 al-1 a* (isolates number 5, 7 and 12; all obtained from THAD PITTENGER, Kansas State University) and [II, +] 237 *nic-1 al-2 a*. These heteroplasmons were started in "race" tubes by PITTENGER. The H3 set consisted of 36 heteroplasmons formed between [I, +] 7 *pan-2 al-1 a* (above) and isolates of a cross between [II, +] 56 *pan-2 al-2 A* and [IIa, poky] PP-6 *nic-1 al-2 a* with the [poky] strain used as the maternal parent. Ten hybrids were formed between the [I, +] strain and individual [poky] isolates from the cross, and the remaining 26 hybrids were formed between the [I, +] parent and a single [IIa, poky] *nic-1 al-2 a* isolate. The two subsets gave essentially the same results.

Culture conditions and formation of heterokaryons: Maintenance of strains and growth of mycelia were as described previously (LAMBOWITZ and LUCK 1976; MANNELLA and LAMBOWITZ 1978). Heterokaryons were formed by superimposing approximately equal amounts of conidia from each parental strain on solid medium. All heterokaryons of sets H1 and H2 and 29 of 36 heterokaryons of set H3 were formed between strains having complementing vitamin requirements. These heterokaryons were grown on minimal medium. The remaining seven heterokaryons of the H3 set were grown on minimal medium supplemented with pantothenate (10 μg per ml) and nicotinamide (10 μg per ml). For the H1 set, heterokaryosis was confirmed by recovery of colonies containing the individual *pan*- and *nic*- nuclei after plating conidia on minimal medium supplemented with both pantothenate and nicotinamide (MANNELLA and LAMBOWITZ 1978). For the H2 and H3 sets, parental strains contained complementing albino markers so that formation and maintenance of heterokaryons could be assessed by pigmentation of the cultures. Heterokaryons started in race tubes were sampled at the hyphal front after various lengths of growth. Sampled cells were recultured to obtain mass amounts of conidia and then used to inoculate liquid cultures for biochemical experiments.

Isolation of mitochondria: Mitochondria were isolated by the flotation gradient method (LIZARDI and LUCK 1971) with modifications (LAMBOWITZ 1979).

Isolation of mitochondrial nucleic acids: Mitochondrial RNA and DNA were isolated from nucleoprotein pellets (LAMBOWITZ and LUCK 1976) as described previously (MANNELLA and LAMBOWITZ 1978). In some experiments, mitochondrial RNA and DNA were extracted directly

from gradient purified mitochondria by SDS-urea-phenol treatment and separated on hydroxy-apatite columns according to STEPIEN et al. (1978).

Restriction endonuclease digestion and gel electrophoresis: MtDNA was digested with restriction endonuclease Eco RI using reaction conditions specified by the supplier (Miles Laboratories). Restriction enzyme digests were analyzed by electrophoresis on agarose or polyacrylamide gels. For routine analysis, 4 to 6 μg DNA were electrophoresed on vertical slab gels, 21 cm in the electrophoresis direction. The gels contained either 0.8% agarose (3 cm thick) or a linear gradient of 4 to 9% polyacrylamide (1.5 mm thick) in a buffer containing 90 mM boric acid, 90 mM Tris and 2.5 mM EDTA. Electrophoresis on agarose gels was at 45 V for 16 hr and on polyacrylamide gels at 30 mA for 18 to 20 hr. The gels were stained with ethidium bromide (4 μg per ml) and photographed under short wavelength UV light.

RESULTS AND DISCUSSION

The parental mtDNA types used in the present study provide four physical markers that can be scored simultaneously in Eco RI digests (Figure 1). Types I and II mtDNA have been described previously by BERNARD et al. (1976). For ease of presentation, the differences between these two mtDNAs are represented as insertions, one of 1200 bp in type II Eco RI-5 and the other 50 bp in type I Eco RI-9 (Figure 1). The [poky] mutant was derived from the Lein 7A wild-type strain, which contains type II mtDNA (see genealogy in MANNELLA, PITTENGER and LAMBOWITZ 1979). Most [poky] strains contain type II mtDNA, but several related subcultures have been found to contain a variant mtDNA, designated type IIa (MANNELLA and LAMBOWITZ 1978; MANNELLA, PITTENGER and LAMBOWITZ 1979). The variant mtDNA contains tandem head-to-tail reiterations of unit length 2100 bp, occurring at variable frequency near the junction of Eco RI-4 and 6; the reiterations are readily detected as a novel band (α, Mr $=$ 1.4 Mdal) in Eco RI digests (MANNELLA, GOEWERT and LAMBOWITZ 1979). Type IIa mtDNA is also characterized by an alteration in Eco RI-10 which can be detected by increased mobility of this fragment on polyacrylamide, but not on agarose gels
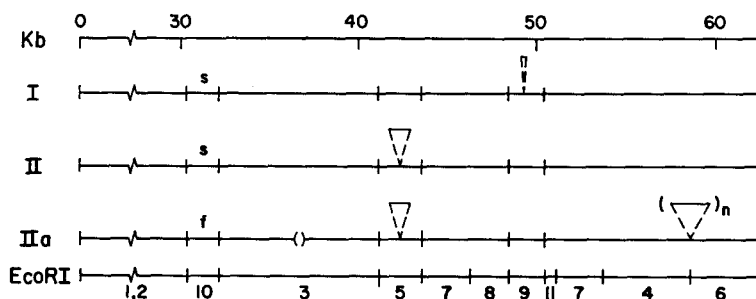

FIGURE 1.—Physical markers present in Neurospora mtDNAs. From left to right, Figure shows Eco RI-10, slow and fast migrating forms (s or f), insertions in Eco RI-5 and 9, and tandem head-to-tail repeats at the junction of Eco RI-4,6 (*i.e.*, α) (see Text). For type IIa mtDNA, the figure also shows small deletions in Eco RI-3 detected in Pst I digests or by redigestion of Eco RI-3 with Hpa II (MANNELLA, GOEWERT and LAMBOWITZ 1979). Eco RI map is based on BERNARD, GOLDTHWAITE and KUNTZEL (1976) and TERPSTRA, HOLTROP and KROON (1977).

(MANNELLA, GOEWERT and LAMBOWITZ 1979; MANNELLA, PITTENGER and LAMBOWITZ 1979).

In the experiments comprising the present study, heteroplasmons were formed between three different sets of parental strains containing different mtDNA types. After subculturing to permit recombination and segregation of mtDNA species, mtDNAs of hybrid strains were analyzed by digestion with Eco RI. Figures 2 and 3 show that recombinants can be readily identified as nonparental mtDNA types with markers derived from both parents. At the same time, mixtures are distinguished by heterogeneity of markers (see Figure 2) and by variations in the ratios of unresolved markers with continued subculturing. In nearly all cases, mixtures were found to be resolved after several conidial passes, reflecting rapid segregation of mtDNA species by some unknown mechanism. Table 1 shows that only five of 44 hybrid strains contained mixtures of mtDNAs and that only one of these remained unresolved at more than one marker.

The 44 mycelial hybrids summarized in Table 1 include 36 formed between parents containing [I, +] and [IIa, poky] mtDNAs, the combination that provides the greatest number of markers. Considering the physical markers first, the following points can be drawn from the data in Table 1:

(1) In heteroplasmons in which type I mtDNA is present with type II or IIa mtDNA, the parental type I mtDNA was never recovered, whereas the parental types II or IIa mtDNA were recovered in about one-third of the total subcultures.
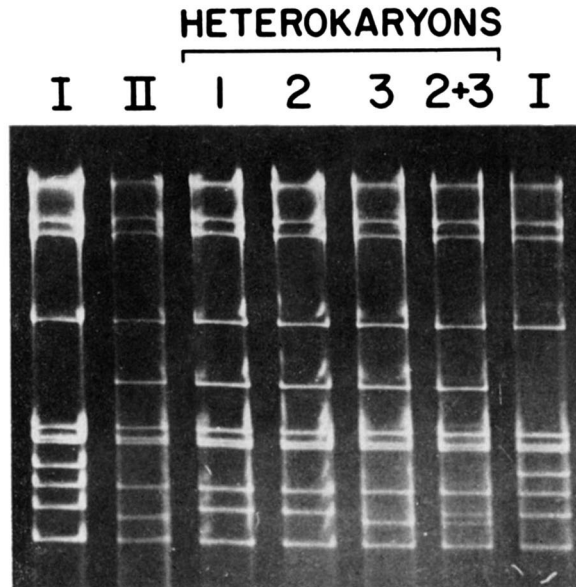

FIGURE 2.—Eco RI digests of mtDNAs from hybrid set H2. I and II are mtDNAs from the parental strains; 1 to 3 are mtDNAs from hybrid strains; 2 + 3 shows co-electrophoresis of mtDNAs from hybrids illustrating resolution of the two forms of Eco RI-9 (second band from the bottom). The gel contained 0.8% agarose. The direction of electrophoresis was from top to bottom.
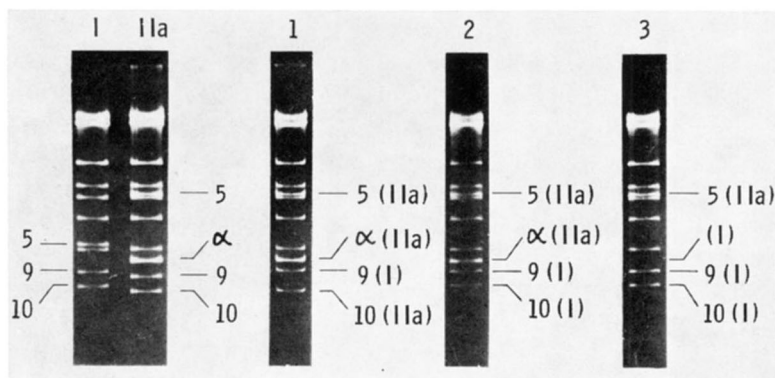
FIGURE 3.—Eco RI digests of mtDNAs from representative H3 hybrids. I and IIa are mtDNAs from the parental strains; 1 to 3 show hybrid strains illustrating three of the four observed recombinant types: 1, IIa-IIa-I-IIa; 2, I-IIa-I-IIa; and 3, I-IIa-I-I using the order Eco RI 10-5-9-α (Table 1). The gel contained a linear gradient of 4 to 9% polyacrylamide. The direction of electrophoresis was from top to bottom.

TABLE 1

Physical marker patterns of the mtDNAs of different Neurospora heteroplasmon sets

| Heteroplasmon set | Parents | No. examined | EcoRI band | | | α | DNA type* | No. |
			10	5	9			
H1	[II, +] ×	5	II			II	P	3
	[IIa, poky]		IIa			IIa	P	1
			II			II/IIa	P/R	1†‡
H2	[I, +] ×	3		II	II		P	1
	[II, +]			II	I		R	2
H3	[I, +] ×	36	IIa	IIa	IIa	IIa	P	9§
	[IIa, poky]		IIa	IIa	I	IIa	R	5
			I	IIa	I	I	R	16‖
			I	IIa	I	IIa	R	1
			IIa	IIa	I	I	R	1
			I/IIa	IIa	I	IIa	R	1†
			I/IIa	IIa	I	I	R	1†
			I	IIa	I/IIa	I	R	1†
			I/IIa	IIa	I/IIa	IIa	P/R	1†

* Parental and recombinant mtDNA types are indicated by P and R, respectively. Refers to physical markers only.

† Presumed to contain a mixed population of mtDNAs, unresolved at markers indicated.

‡ Corresponds to heterokaryon #8 of MANNELLA and LAMBOWITZ (1978) in which α has intensity of singlet rather than triplet. Further analysis indicated that α is still present as a variable frequency repeat in some molecules in the preparation, but that the concentration of α repeats is decreasing with continued subculturing.

§ One of these subcultures displayed non-[poky] phenotype.

‖ Three of these subcultures displayed non-[poky] phenotype.

(2) In all, recombinant mtDNA types were recovered in about two-thirds of the subcultures. However, the number of different recombinant types was surprisingly small. In the H3 set, involving types I and IIa mtDNA, only four of the 14 possible recombinant types were observed. Two additional recombinant types are added if we include those that may be (but are not necessarily) present in the mixtures indicated in Table 1.

(3) The predominant pattern of recombination can be represented as unidirectional gene conversion with respect to two markers: the 1200 bp insertion in Eco RI-5 (II) and the 50 bp insertion in Eco RI-9 (1). Two recombinant types, I-IIa-I-I and IIa-IIa-I-IIa (using the order Eco RI 10-5-9-α) are by far the most frequent, occurring 16 and 5 times, respectively (Table 1; Figure 3 shows gel patterns for recombinant types). The digest patterns for the recombinants give no indication that the insertions occur anywhere but at their original sites in Eco RI-5 and 9. Strictly speaking, however, we cannot yet exclude the possibilities that they can occur at different sites within these fragments or that they can "move" to different fragments at low frequency.

(4) The efficiency of execution is particularly striking for the 1200 bp insertion, which was recovered in all 44 mycelial hybrids containing either parental or recombinant mtDNA types (Table 1). The 50 bp insertion was found in all subcultures containing recombinant type mtDNAs; it is missing only in subcultures containing the parental types II or IIa mtDNA. In addition, all subcultures were found to become rapidly homoplasmic with respect to the 1200 bp insertion, whereas two subcultures remained unresolved with respect to the 50 bp insertion. The high efficiency of execution for the 1200 bp insertion presumably accounts for the failure to recover the parental type I mtDNA in any subculture.

(5) An additional interaction between markers: Eco RI-10 (IIa) shows surprisingly tight linkage to α (about 95%), perhaps reflecting a causal connection as discussed in detail previously (MANNELLA, GOEWERT and LAMBOWITZ 1979).

The behavior of the four physical markers constitutes the first half of the description of events in the heteroplasmons. The second half centers on the transmission of the [poky] mutation which is carried on type IIa mtDNA in the H1 and H3 hybrid sets. In these two sets, 32 of the 41 hybrids were scored for mitochondrial phenotype both by growth rate and by ratios of 19 S to 25 S mitochondrial rRNAs as described previously (MANNELLA and LAMBOWITZ 1978). Initially, the hybrids showed the wild-type phenotype but with continued subculturing growth rate decreased and the mycelia became [poky]. At the time the experiments were terminated, 28 of the 32 subcultures scored for phenotype had become [poky] by the two criteria cited above, while only two remained wild type and two more showed intermediate phenotypes. It should be noted that each of the four non-[poky] hybrids had undergone minimal subculturing (two to five conidial passes) consistent with the earlier observation that upwards of eight conidial passes are sometimes required for expression of the [poky] phenotype in such hybrids (MANNELLA and LAMBOWITZ 1978). The physical marker patterns of the mtDNAs of the non-[poky] hybrids are indicated in Table 1.

Table 2 summarizes our attempt to determine whether the [poky] mutation might be linked to any of the four physical markers. It can be seen that [poky] shows essentially no linkage to any of the physical markers, with the possible exception of Eco RI-5 (II). In the latter case, however, the apparent linkage seems to reflect only the simultaneous recovery of both markers as judged by the following evidence:

(1) [poky] is "dominant" even when both the wild-type and [poky] parental mtDNAs contain the Eco RI-5 (II) segment (hybrid set H1, Table 1; also MAN-NELLA and LAMBOWITZ 1978).

(2) Eco RI-5 (II) spreads through mtDNA populations even when both parental mtDNAs are wild type (hybrid set H2, Table 1; however, data limited to three subcultures).

(3) The spread of Eco RI-5 (II) through mtDNA populations appears to be more rapid than that of the [poky] mutation. The H2 hybrid strains, started in "race" tubes and sampled after 1 cm of growth (*i.e.*, one conidial pass; see MA-TERIALS AND METHODS) had already become homoplasmic for Eco RI-5 in contrast to the eight or more conidial passes sometimes required for expression of the [poky] phenotype. In addition, the four hybrids in the H3 set that were analyzed before they expressed the [poky] phenotype (see above) were found to have already become homoplasmic for Eco RI-5 (IIa). The latter result shows directly that transmission of Eco RI-5 (IIa) is not directly correlated with the expression of the [poky] phenotype.

In summary, therefore, we have identified two insertions in Neurospora mtDNA that appear to be sites of high frequency, unidirectional gene conversion as judged by their spread through mtDNA populations in heteroplasmons. However, neither insertion could be correlated with the [poky] mutation. In essence, the behavior of the Neurospora insertions appears analogous to that described previously in yeast for the 1000 bp ω^+ insertion (COEN *et al.* 1970; DUJON and SLONIMSKI 1976; JACQ *et al.* 1977; SANDERS *et al.* 1977) and more recently for several smaller insertions (less than 60 bp) in the *var-1* region (STRAUSBERG *et al.* 1978). In the case of the ω^+ insertion, the gene conversion event has also been shown to lead to preferential recovery of closely linked flanking markers, with a gradient of decreasing efficiency for markers more distal to the insertion (*i.e.*, polar recombination; COEN *et al.* 1970; DUJON and SLONIMSKI 1976). This point

TABLE 2

Apparent linkage between [poky] lesion and mtDNA markers

Marker location	Linkage with [poky]*
EcoR-10	56%
EcoRI-5	92%
EcoRI-9	38%
EcoRI-4/6 (α)	61%

* Linkage is defined as the percent of hybrid strains in which the two markers are resolved and derived from the same parental mtDNA.

cannot be tested for the Neurospora insertions because of the lack of suitable closely linked flanking markers. Mechanisms that could account for unidirectional gene conversion associated with mtDNA insertions include intracellular selection, heteroduplex pairing and repair, and site specific recombination (DU-JON et al. 1974; PERLMAN and BIRKY 1974; STRAUSBERG et al. 1978; VAN WINKLE-SWIFT and BIRKY 1978). In terms of a transposon mechanism, it might be of interest to look for homology between the yeast and Neurospora insertions. It could also prove illuminating to compare the location of insertions with respect to known genetic markers in the two types of mtDNA. In yeast, the ω^+ insertion is located within the gene encoding the 21 S (large) mt rRNA (Bos et al 1978; FAYE et al. 1979), whereas both Neurospora insertions are located opposite the rRNA genes (BERNARD et al. 1976; TERPSTRA, HOLTROP and KROON 1977). It should be noted, however, that the gene encoding the 25 S rRNA in Neurospora mitochondria has been found to contain an intron of 2.3 kb located ca. 500 bp from one end of the gene, as is the ω^+ insertion in the yeast 21 S rRNA gene (HAHN et al. 1979). In light of our results, it is possible that the Neurospora 25 S rRNA intron would show unidirectional gene conversions analogous to that of the yeast ω^+ intron in heteroplasmons with mtDNAs lacking the intron.

Our results show that unidirectional gene conversion is one mechanism that could account for the "dominance" of [poky] and other extra-nuclear Neurospora mutants in heteroplasmons. Since Neurospora extranuclear mutants have always been selected by procedures that favor "dominant" mutations and since "dominance" appears to be associated with insertions, it is possible that [poky] and other extranuclear Neurospora mutants contain small insertions potentially detectable by restriction-enzyme analysis. However, the data do not exclude other models for "dominance," such as a more rapid replication of mutant mitochondria or mtDNA that could occur even in the absence of large mtDNA alterations. The frequent occurrence of loci showing unidirectional gene conversion greatly complicates the mapping of Neurospora extranuclear mutants using the available physical markers. Our previous studies raised the possibility that the primary defect in [poky] might be in the mitochondrially synthesized, mitochondrial ribosomal protein, S-5 (LAMBOWITZ, CHUA and LUCK 1976). We are presently taking a biochemical approach: attempting to identify alterations in the primary structure of S-5 in [poky] and other extranuclear Neurospora mutants with similar phenotypes.

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