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INTERACTION OF WILD-TYPE AND POKY MITOCHONDRIAL DNA IN HETEROKARYONS OF NEUROSPORA

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SUMMARY: The mitochondrial phenotype of poky and other extra-nuclear Neurospora mutants is known to predominate over that of wild type in heteroplasms. This phenomenon was investigated in heterokaryons using normally occurring strain differences in restriction enzyme patterns to distinguish wild-type and poky mitochondrial DNAs. Each of ten independent heterokaryons eventually showed the poky phenotype as judged by slow growth rate and deficiency of 19 S RNA. Six heterokaryons contained mitochondrial DNAs with restriction enzyme patterns of the poky parent whereas four contained DNAs which lacked restriction enzyme fragments characteristic of the poky parent. The latter may be recombinants of wild-type and poky mitochondrial DNA.

Studies by Pittenger (1) and Diacumakos et al. (2) showed that the mitochondrial phenotype of poky (mi-1) and other extra-nuclear Neurospora mutants predominates over the wild-type phenotype in heteroplasms formed by heterokaryosis or by microinjection of mutant mitochondria. The mechanism of "dominance" is not understood, but the phenomenon can be distinguished from the suppressiveness of yeast petite mutants by the lack of gross structural alterations associated with Neurospora mit* DNA (3,4). Tatum and Luck (5) suggested that "dominance" might reflect a replicative advantage of the mutant over the wild-type genetic determinant (presumably mit DNA) whereas other authors have drawn attention to virus-like particles found in mutant cells (6). Until now "dominance" has been difficult to investigate because of the paucity of genetic markers on Neurospora mit DNA. Recent results, however, show that

*Abbreviations: mit, mitochondrial; Mdal, megadaltons; pan, pantothenic acid; nic, nicotinic acid; Eco RI, restriction endonuclease RI from E. coli.

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the mit DNAs of even closely related fungal strains possess sufficient sequence diversity to be distinguished by restriction enzyme analysis (3,7,8). By selecting appropriate parent strains, we were able to use such normally occurring differences as markers for wild-type and poky mit DNAs in mixed cytoplasms. The present report describes the first use of this experimental system to investigate whether "dominance" is due to a replicative advantage of mutant mit DNA.

METHODS

Strains of Neurospora crassa were [+] 46 a pan-2, [+] 237 a nic-1 al-2, [poky] PU-5 a pan-2 and [poky] PP-6 a nic-1 al-2**; all strains were obtained from Drs. H. Bertrand and R. Collins of the University of Regina. The strains possessed similar nuclear backgrounds except for the vitamin requirements (pantothenic and nicotinic acids). Heterokaryons were formed between wild-type and poky parents by superimposing conidia of strains with complementing vitamin requirements on minimal medium. The cells were subcultured until the growth rate decreased, i.e., after 2 to 8 conidial passes (3 to 12 weeks). The time required appeared unrelated to the initial ratios of wild-type and poky conidia used in forming the heterokaryons. Heterokaryosis was confirmed by recovery of the individual pan^- and nic^- nuclei from colonies grown on minimal medium supplemented with both pantothenic and nicotinic acids. Maintenance of strains, growth of mycelia and isolation of mitochondria were carried out as described previously (9).

Mitochondrial nucleoprotein pellets were obtained by lysis of mitochondria in Ca^{+2}-containing buffer followed by centrifugation of the lysates through a cushion of 1.85 M sucrose (9). For the isolation of nucleic acids, the pellets were resuspended in 2 ml of NET buffer (150 mM NaCl, 1 mM EDTA, 100 mM Tris·HCl, pH 8.2) containing 1% sodium dodecylsulfate (supplemented with 4% (v/v) diethylpyrocarbonate for RNA isolation) and extracted twice with an equal volume of NET-saturated, redistilled phenol. Mit RNA was precipitated from the aqueous phase by the addition of 2.5 volumes of ethanol and storage at -20°C. Electrophoretic analysis of RNAs was carried out as described previously (9). For the isolation of mit DNA, the aqueous phase was dialyzed overnight in the cold against NET buffer. The dialysate was incubated at 37° for 30 min with RNase (8 units Sigma RNase A Type XII-A and 240 units Sigma RNase T_1, Grade III, preheated in 50 µl NET buffer for 10 min at 80°C), then for 40 min with protease (2.5 units, Sigma Protease Type VI, preincubated for 60 min at 37°C in 200 µl NET) and finally extracted with phenol. The aqueous phase was dialyzed extensively against 150 mM NaCl, 0.1 mM EDTA, 10 mM Tris·HCl, pH 7.5. Mit DNA was ethanol-precipitated from the final dialysate and resuspended in 10 mM NaCl, 0.1 mM EDTA, 10 mM Tris·HCl, pH 7.5. This procedure gave 100 to 200 µg mit DNA per ml of packed mitochondria. Restriction enzyme digestion and electrophoretic analysis was carried out essentially as described by Murray and Murray (10), but using 0.8% agarose gels containing 90 mM boric acid, 2.5 mM EDTA and 90 mM Tris. Gels were stained with ethidium bromide (4 µg per ml) and photographed under short wavelength UV light.

**The mitochondrial phenotype of each strain is indicated in square brackets; [+] indicates wild-type.

RESULTS AND DISCUSSION

Fig. 1 illustrates the Eco RI fingerprints of mit DNAs from the wild-type and poky parents used in the present study. The pattern for the wild-type parents is the same as that described previously for several other wild-type strains including Lein 7A (3). This pattern is also characteristic of most poky strains in our laboratory, as expected since poky was derived from Lein 7A (M. Mitchell, personal communication). In screening many strains, however, poky strains were identified which displayed the altered pattern shown in Fig. 1. This pattern is characterized by one additional band (M_r ~1.25 Mdal), presumably reflecting a spontaneous alteration which occurred in an antecedent strain. The additional Eco RI band appears to be a triplet as judged by microdensitometry of gel photographs like that of Fig. 1 and of autoradiographs of gels containing ^{32}P-labeled DNA. Preliminary experiments suggest further that the three fragments occur in tandem and represent a reiteration of a nearby region of the mitochondrial genome (Mannella and Lambowitz, manuscript in preparation). Since the extra Eco RI triplet does not appear to be correlated with the expression of the poky gene, it was considered an independent, physical marker for the mit DNA of the poky parent in the present study.

Figs. 1 and 2 show results for ten independent heterokaryons, #1-5 formed between [+] pan$^-$ and [poky] nic$^-$ parents and #6-10 formed between [+] nic$^-$ and [poky] pan$^-$ parents. The heterokaryons were distinguished as wild-type or poky on the basis of growth rates and ratios of 19 S to 25 S mit rRNA. As indicated in "Methods", all ten heterokaryons showed a decreased growth rate after some period of subculturing. Fig. 2 shows that, in each case, the decreased growth rate could be correlated with the gross deficiency of 19 S mit rRNA characteristic of the poky phenotype (9,11,12). At the same time analysis of the mit DNAs gave surprising results. As shown in Fig. 1, six of the heterokaryons contained mit DNAs with the extra Eco RI triplet characteristic of the poky parent but three heterokaryons (#6, 9 and 10) con-

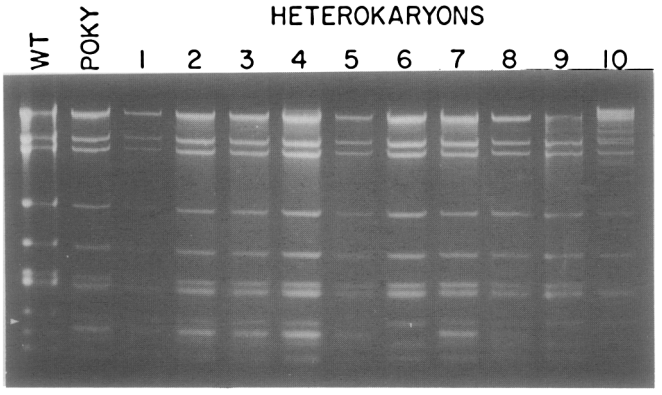

FIGURE 1 Gel electrophoresis of Eco RI digests of mit DNAs isolated from [+] pan⁻ (WT) and [poky] pan⁻ parent strains, and from ten heterokaryons. The direction of electrophoresis is from top to bottom. The arrow at the left indicates the position of the 1.25 Mdal band. The reaction of Eco RI with the mit DNA of heterokaryon 10 was incomplete, as judged by the partial digest bands in the high molecular weight region of the gel. Subsequent gel electrophoresis of limit digests of the same mit DNA confirms the absence of a DNA band in the 1.25 Mdal region.

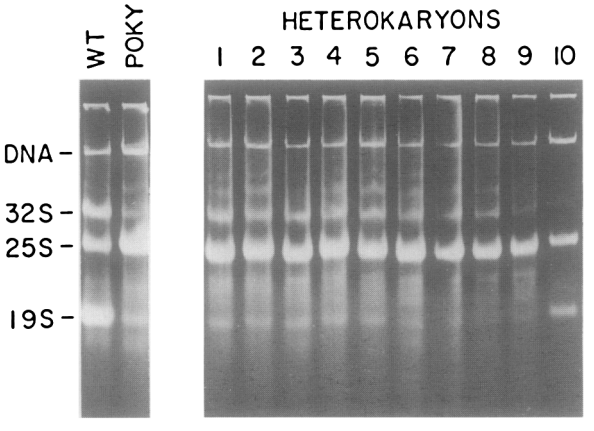

FIGURE 2 Gel electrophoresis of nucleic acids isolated from the mitochondria of [+] pan⁻ (WT) and [poky] pan⁻ parent strains and of ten heterokaryons. The direction of electrophoresis is from top to bottom.

tained mit DNAs which lacked this band completely and one heterokaryon (#8) contained mit DNA in which the band was a singlet rather than a triplet. The "singlet" pattern was found to be unchanged after seven additional conidial passes, the long-term stability arguing against the possibility that hetero-

karyon #8 merely contains a 2:1 mixture of wild-type and poky mit DNAs. It may be significant that all four of the anomalous mit DNAs arose in heterokaryons obtained from the [+] nic$^-$ and [poky] pan$^-$ parents. Although it is possible that the Eco RI triplet is unstable in this particular combination, it should be noted that the mit DNA of heterokaryon #7, the remaining isolate from this combination, did contain the Eco RI triplet at the time of the experiment of Fig. 2 and retained it after seven additional conidial passes (an additional 12 weeks).

The anomalous mit DNAs in heterokaryons #6 and #8-10 are not compatible with a simple "replicative advantage" model. Indeed, while the results confirm that the poky phenotype prevails in heterokaryons, they also raise the possibility that the interaction between wild-type and mutant mit DNAs may be more complicated than was previously believed. There are at least 4 possible explanations for the anomalous heterokaryons: (a) that the poky gene is not carried on mit DNA, (b) that an undetectably small amount of poky mit DNA is sufficient for the expression of the poky phenotype, (c) that the Eco RI triplet is excised from poky mit DNA at high frequency and (d) that the anomalous heterokaryons contain mit DNAs which retain the poky gene but have lost restriction fragments by recombination with wild type. There is no independent evidence in favor of possibilities (a) and (b) and the persistence of the 1.25 Mdal band in the mit DNAs of heterokaryons #7 and #8, even after subculturing, tends to argue against the excision mechanism (c). On the other hand, the recombination hypothesis (d) is favored by results with yeast and Chlamydomonas which suggest that mit and chloroplast DNAs can recombine at high frequency and that mechanisms exist for the rapid segregation of different DNA species (13,14). If the four anomalous heterokaryons do contain recombinants of wild-type and poky mit DNA, then the results are consistent with the idea that the poky gene confers some replicative advantage on mit DNA in heterokaryons. Experiments are in progress to verify the identity of the putative recombinants by locating additional markers in other regions

of mit DNA using restriction enzyme analysis. Restriction enzyme analysis of the recombinant molecules may also be useful in mapping poky and other extranuclear mutations on mit DNA. If the anomalous heterokaryon mit DNAs represent recombinant molecules, the fact that all four occurred with the [+] nic^- and [poky] pan^- parents suggests that recombination pathways might be exceptionally active in one or both of these strains. Obviously, a much larger sampling of heterokaryons is needed to test this possibility.

An interesting finding of the present work is that poky strains which were derived from the same parent contain different molecular species of mit DNA. The 1.25 Mdal fragments presumably reflect a spontaneous insertion which occurred during subculture without affecting the poky phenotype. Sanders et al. (8) have studied an AT-rich region of yeast mit DNA which appears to have variable length in different yeast strains. The mapping experiments cited previously (Mannella and Lambowitz, manuscript in preparation) tentatively locate the 1.25 Mdal fragments in an AT-rich region of Neurospora mit DNA (between Eco RI fragments C and K, according to the nomenclature of Bernard et al. (3)).

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