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Premature Death in *Podospora anserina:* Sporadic Accumulation of the Deleted Mitochondrial Genome, Translational Parameters and Innocuity of the Mating Types

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ABSTRACT

The *Podospora anserina* premature death syndrome was described as early growth arrest caused by a site-specific deletion of the mitochondrial genome (mtDNA) and occurring in strains displaying the genotype *AS1-4 mat−*. The *AS1-4* mutation lies in a gene encoding a cytosolic ribosomal protein, while *mat−* is one of the two forms (*mat−* and *mat+*) of the mating-type locus. Here we show that, depending on culture conditions, death due to the accumulation of the deleted mtDNA molecule can occur in the *AS1-4 mat+* context and can be delayed in the *AS1-4 mat−* background. Furthermore, we show that premature death and the classical senescence process are mutually exclusive. Several approaches permit the identification of the *mat*-linked gene involved in the appearance of premature death. This gene, *rmp,* exhibits two natural alleles, *rmp−* linked to *mat−* and *rmp+* linked to *mat+*. The first is probably functional while the second probably carries a nonsense mutation and is sporadically expressed through natural suppression. A model is proposed that emphasizes the roles played by the *AS1-4* mutation, the *rmp* gene, and environmental conditions in the accumulation of the deleted mitochondrial genome characteristic of this syndrome.

DELETIONS of the mitochondrial genome (mtDNA) can occur spontaneously in most, if not all, eucaryotes. Such a case is well-known in the yeast *Saccharomyces cerevisiae* where the *rho*⁻ mutants were demonstrated for the first time more than 40 years ago (EPHRUSSI 1953; FAYE *et al.* 1973). These deletions have been more recently documented in higher eucaryotes, especially humans (see POULTON 1992; WALLACE 1992; SHAPIRA 1993 for reviews). Obligate aerobes can carry deleted mitochondrial genomes (ΔmtDNAs) only in a heteroplasmic state in which a threshold level of wild-type mtDNA ensures cell survival. In humans, a number of sporadic diseases have been described for which the causative agent is a ΔmtDNA that has competitively minimized the wild-type genome in the affected tissues. Such diseases are currently explained by a spontaneous deletion occurring in early development, followed by clonal expansion of the deleted molecule. They are not linked to any obvious family history and, although the deleted molecule can vary between patients, each harbors a single species of ΔmtDNA (see reviews cited above). These deleted molecules have been found at very low levels (observable only by PCR) during normal aging (see WALLACE 1992 for a review). One class of these ΔmtDNAs has also been found in human oocytes (CHEN *et al.* 1995). These data raise a fundamental and unresolved problem. Do conditions, either environmental or due to somatic mutations, exist that can increase the rate of mtDNA deletions and/or result in the deleted molecules populating some tissues at the expense of the wild-type genome? These questions are difficult to address in humans, although some nuclear genes are clearly involved in some diseases linked to mtDNA deletions (see WALLACE 1992; POULTON 1992; SCHAPIRA 1993 for reviews). However, the roles of these genes in the appearance and transmission of deleted mtDNAs remain unknown.

One approach to the problems of mtDNA stability and mtDNA transmission during mitotic divisions addresses the questions in lower eucaryotes, more amenable than metazoa to classical and molecular genetics. In *S. cerevisiae,* a number of genes have been identified that either increase the rate of the *rho*⁻ mutations or control the relative transmission of the deleted and wild-type molecules in heteroplasmons (see DISCUSSION for references). However, while yeasts are powerful model systems, they exhibit intrinsic weaknesses with respect to our purpose: they are facultative aerobes and they are unicellular. Filamentous fungi are alternate and complementary models since they are obligate aerobes on the borderline between unicellular and multicellular organisms. Mitochondrial DNA rearrangements leading to cell death (senescence) are well documented in a few of these fungi (GRIFFITHS 1992, for a review). However, to our knowledge, only two cases of nuclearly controlled ΔmtDNA accumulation

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have been reported: natural death in *Neurospora crassa* (SEIDEL-ROGOL *et al.* 1989; BERTRAND *et al.* 1993) and premature death in *Podospora anserina* (BELCOUR *et al.* 1991; DEQUARD-CHABLAT and SELLEM 1994; SELLEM *et al.* 1993).

Premature death was described as a growth arrest that occurs very early after ascospore germination. It is probably due to accumulation of a single class of deleted mtDNA molecules, under the control of two nuclear genes. One is a mutant allele (*AS1-4*) of a gene encoding a cytosolic ribosomal protein (DEQUARD-CHABLAT *et al.* 1986; DEQUARD-CHABLAT and SELLEM 1994) and the other is associated with the *mat*− haplotype. For these reasons, premature death syndrome was described as a specific feature of the *AS1-4 mat*− genotype (BELCOUR *et al.* 1991). Deletion is assumed to arise from an intramolecular recombination event between two direct repeats. The target sequence is a mobile intron that can occupy a specific ectopic site other than its natural location: recombination between the two copies of this intron should give rise to two complementary deleted molecules, one of which is lost (SELLEM *et al.* 1993). This syndrome was observed only in *AS1-4 mat*− strains, while *AS1-4 mat*+ strains appeared immortal (BELCOUR *et al.* 1991). Furthermore, although showing the site-specific deletion at an extremely low level (C. SELLEM, personal communication), the wild-type strains ($AS1^+$ *mat*+ and $AS1^+$ *mat*−) do not appear to accumulate the deleted molecule. Instead, they display a senescence syndrome associated with other mtDNA rearrangements: short mtDNA sequences are amplified as circular multimeric DNA molecules (called senDNAs) in senescent cultures (see DUJON and BELCOUR 1989 for review). Strikingly, the sequence most often amplified (senDNAα) is exactly that which plays a key role in the recombinational event leading to premature death (BELCOUR *et al.* 1991).

Further analyses were required to better understand premature death syndrome. The physiological and genetic approaches reported in this paper yield two main conclusions. First, the syndrome can no longer be defined simply as an early growth arrest but rather as the accumulation of a specific mtDNA deletion. This can occur in both *AS1-4 mat*− and *AS1-4 mat*+ strains depending upon culture conditions. Second, the mating-type genes do not play any role in the process. There is a distinct gene, *rmp*, with two natural alleles, *rmp*+ and *rmp*−, linked respectively to *mat*+ and *mat*− (*rmp* is a french acronym for "régulateur de la mort prématurée," SELLEM *et al.* 1993). Overall, the data suggest a model that takes into account the fact that senescence and premature death are mutually exclusive syndromes and that highlights the utmost importance of cytosolic translation within a syndrome due to the accumulation of a specifically deleted mitochondrial genome.

MATERIALS AND METHODS

Fungal strains: Genetics and biological properties of *P. anserina* were first described by RIZET and ENGELMANN (1949) and reviewed by ESSER (1974). The asci contain four ascospores, each formed around two nonsister nuclei after a postmeiotic mitosis. A few asci contain five ascospores, two of which are smaller and uninucleate. These give rise to homocaryotic mycelia, mainly used for genetic analysis. When necessary, entire asci were investigated. All mutant strains are derived from the S strain (RIZET 1952). The *mat* locus maps to linkage group I (LGI) and displays 98% second division segregation (SDS). The *AS1-4* and *AS3-2* mutations were identified as antisuppressor mutations (PICARD-BENNOUN 1976). The first is in LGI and the second in LGIV. The *su8-1* mutation is a UGA tRNA suppressor (DEBUCHY and BRYGOO 1985) displaying nearly 100% first division segregation (FDS). The *PmA-1* mutation is a *mat*-linked marker resulting in increased sensitivity to paromomycin and small-sized spores (MARCOU *et al.* 1980). The *lys2-1* and *leu1-1* mutants that, respectively, exhibit auxotrophy for lysine and leucine are gifts of M. CROUZET. The strain carrying both a deletion of the *mat* locus and transgenic *mat*− information was described by COPPIN *et al.* (1993). The geographical races A, M, N, s, U, W, Z and the *P. comata* species (T strain) are kind gifts of G. RIZET and D. MARCOU. Their incompatibility properties with respect to the S race were investigated either by RIZET (1952), BERNET (1965), or L. BELCOUR (unpublished results). The lifespans of the A, M, N, and S races were described by MARCOU (1961), while the mtDNA characteristics of all races were investigated either by CUMMINGS *et al.* (1990) or L. BELCOUR (unpublished results).

Media, growth rate and lifespan measurements: All media, *i.e.*, corn meal extract (MR), minimal synthetic (M2) and germination (G) media were as described by ESSER (1974). When necessary, media were supplemented with drugs after sterilization. For at least four subcultures of each relevant strain, growth rates were measured as the increase in diameter of the thalli at regular time intervals until the thalli covered the Petri dish. Lifespans were measured for either four subcultures from five strains or five subcultures from four strains exhibiting a given genotype. Exceptionally, four subcultures from three strains were used. Parallel cultures were grown in culture (race) tubes at 26° in the dark. Incubation of tubes and transfers were made with implants of ~10 mm^2. The lifespan of a strain was defined as the mean length of growth of parallel cultures between the point of incubation and the arrested edge of the dead culture. Growth arrest was readily detected due to the pigmentation changes of dead mycelium.

Crossing procedures, construction of double mutant strains and forced vegetative heterocaryons: Crosses were performed either by confrontation or spermatization. In the first, the two relevant (homocaryotic) strains were allowed to grow on the same Petri dish, crossing occurring when the two cultures met. In the second, a suspension of microconidia obtained from one strain (the male culture) was poured onto a homocaryotic mycelium (the female culture). This procedure was used either to analyze strains obtained from dicaryotic ascospores or to ensure a precise and common cytoplasmic background in the progeny (for instance, in crosses involving different geographical races). Most double mutant strains were easily obtained and characterized due to their normal germination level and to phenotypic properties of the parental strains that could be unambiguously recovered in the progeny. For instance, the *AS1-4* ascospores display a spindly germinating mycelium not shown by *su8-1* ascospores. The *AS1-4 su8-1* strains behave as *AS1-4* strains at germination. The presence of the *su8-1* mutation was determined by crosses with *193* tester strains carrying a UGA spore color mutation

(PICARD 1973). The *AS1-4 AS3-2* double mutant strains were more difficult to obtain and characterize since *AS3-2* ascospores display a very low germination level, and when germinating, a phenotype that can be confused with that of *AS1-4*. Although the *AS1-4 AS3-2* double mutant strains appeared female sterile (unlike the parental strains), their status was ascertained by crossing each candidate to the wild-type strain. Progeny were analyzed and the segregation of the two mutations was verified. Forced vegetative heterocaryons were obtained by putting implants (one of each of the two relevant auxotrophic strains) side by side on M2 medium, allowing them to form anastomoses and complement one another for growth on this minimal medium.

Transformation procedures and mtDNA analysis: To obtain strains carrying both the wild-type $su8^+$ gene and its *su8-1* mutant allele, transformation experiments were performed as previously described (PICARD *et al.* 1991). The recipient strain carried the UGA *leu1-1* mutation and was $su8^+$ *mat+* (*rmp+*). It was transformed with the pHSU8 vector (DEBUCHY *et al.* 1988) that has the UGA suppressor tRNA *su8-1*, suppressing the *leu1-1* mutation. Several (leu^+) transformants were recovered and crossed to a tester strain carrying the *193* UGA spore color mutation, to ensure that the transgenic *su8-1* suppressor was well expressed. Several transformants were then crossed to a *AS1-4 mat−* (*rmp−*) *leu1-1* $su8^+$ strain. The *AS1-4* (*rmp+* and *rmp−*) progeny were recovered and checked for either a (leu^-) or a (leu^+) phenotype, to identify those with the *su8-1* transgenic information.

Analysis of mtDNA was performed by standard methods (BELCOUR *et al.* 1991) except that the DNA was extracted from mycelia using the rapid procedure described by LECELLIER and SILAR (1994).

RESULTS

Sporadic expression of the premature death syndrome: Preliminary studies showed that *AS1-4 rmp−* strains always displayed mycelial death on the entire growth front of a culture, a few days after ascospore germination (Figure 1A). *AS1-4 rmp+* strains, on the contrary, displayed such a long lifespan that they appeared immortal (BELCOUR *et al.* 1991). At that time, lifespans were measured by growth on corn meal medium (MR) in race tubes (BELCOUR and BEGEL 1980; MATERIALS AND METHODS). Data from experiments in which the lifespans, measured on MR medium, of *AS1-4* strains were compared to those of wild-type ($AS1^+$) strains are shown in Table 1 (lines I and II). As an alternative culture medium, we used minimal synthetic medium (M2), which has been shown to shorten lifespans of $AS1^+$ strains (SILAR and PICARD 1994; this paper, Table 1). Longevities of *AS1-4* (*rmp+* and *rmp−*) strains obtained from different crosses are shown in Table 1 (see lines III–VII 1). These experiments demonstrated two striking points concerning the *AS1-4 rmp+* strains. First, these strains no longer appeared immortal when grown on M2 medium. Second, the lifespans appeared very heterogeneous, as shown by the variations of the mean values between experiments and by the standard deviation within each experiment. These scattered values are not due only to heterogeneity between strains (obtained from different ascospores)

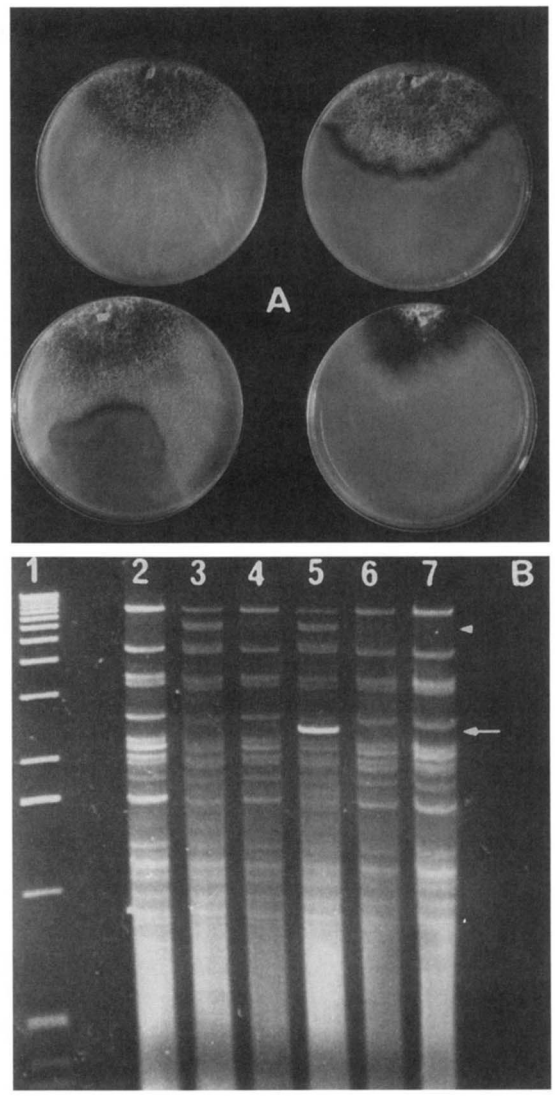

FIGURE 1.—Premature death seen at the levels of mycelial phenotype (A) and mtDNA structure (B). (A) Top: *AS1-4 rmp+* showing either no sign of death (left) or a full edge dead front (right). Bottom left: *AS1-4 rmp+* showing death on a limited area of the growing front. Bottom right: the typical premature death phenotype of a *AS1-4 rmp−* culture. (B) DNA analysis. Lane 1: 1-kb DNA ladder. Other lanes: mtDNA digested by *Hae*III and stained by ethidium bromide from *AS1-4 rmp−* displaying a typical premature death phenotype (lanes 2 and 7, two independent cultures), $AS1^+$ *rmp−* young culture (lane 3), *AS1-4 rmp+* dead after 3 cm growth (lane 4), $AS1^+$ *rmp−* senescent culture (lane 5), *AS1-4 rmp−* first saved from premature death on M2 medium supplemented by paromomycin (two serial Petri dishes), then transferred to M2 medium and having stopped growing after 4 cm (lane 6) (see text for details). Arrow indicates the position of senDNAα (2.5 kb), typical of the senescent state found in wild-type cultures. Arrowhead shows two of the fragments that are underrepresented in mtDNA from mycelia displaying premature death. (Presently, >30 independent premature death events have been analyzed: they all display the same pattern).

but also reflect heterogeneity between different subcultures of the same strain. For example, among four subcultures of a given strain, three died after 2 cm of growth while the fourth stopped growing at 25 cm. This

TABLE 1

Lifespans of the wild-type and *AS1-4* strains grown on different media

Media	Strains			
	AS1⁺ rmp+	*AS1⁺ rmp−*	*AS1-4 rmp+*	*AS1-4 rmp−*
I (MR)	31 ± 7.9 (0.256)	17.2 ± 2.4 (0.139)	>164[a]	ND
II (MR)	36.5 ± 8.5 (0.234)	20.6 ± 5.5 (0.268)	ND	2.8 ± 0.8 (0.290)
III (M2)	8.4 ± 0.5 (0.064)	9.1 ± 0.6 (0.073)	89.3 ± 48.1 (0.538)	1.4 ± 0.3 (0.227)
IV (M2)	9.2 ± 0.7 (0.077)	9.9 ± 0.6 (0.068)	33.4 ± 26.4 (0.791)	ND
V (M2)	9.1 ± 0.7 (0.080)	ND	77.5 ± 50.7 (0.655)	ND
VI (M2)	ND	10.3 ± 2.5 (0.245)	ND	1.7 ± 0.4 (0.230)
VII 1 (M2)	8.6 ± 0.7 (0.081)	10 ± 1.3 (0.137)	124.3 ± 70.5[b] (0.567)	ND
2 (M2 + CHX)	6.7 ± 0.4 (0.067)	8.1 ± 0.6 (0.072)	133.2 ± 62.8[b] (0.471)	ND
3 (M2 + Pm)	10.6 ± 1.4 (0.131)	14.3 ± 2.0 (0.141)	>194[c]	ND
4 (M2 + Ksg)	4.5 ± 0.9 (0.200)	5.4 ± 0.9 (0.178)	>189[d]	ND

Roman numerals refer to different crosses (*AS1-4 rmp+* × *AS1⁺ rmp−*). MR, corn meal medium; M2, synthetic minimal medium; M2+CHX, M2+Pm and M2+Ksg indicate M2 supplemented, respectively, with cycloheximide (2 μg/ml), paromomycin (500 μg/ml) and kasugamycin (500 μg/ml). Mean lifespans are in cm ± SD. Numbers in parentheses are the coefficients of variation (*i.e.*, ratio of standard deviation to mean, SOKAL and ROHLF 1969). Each experiment was performed on 20 subcultures of each relevant genotype, except for the *AS1⁺* strains used in experiments IV and VI (12 subcultures) (MATERIALS AND METHODS). Occasionally mean lifespans were calculated on 14 to 19 subcultures instead of 20 because some were lost to contamination before growth arrest. In experiment VII, explants derived from the same cultures were used in the four conditions in order to ensure the best comparisons. ND, not determined.

[a] Cultures were discontinued before growth arrest.

[b] The values are rough estimates since one culture (among 15) has not yet stopped growing on M2 medium (its lifespan will be over 240 cm) while six cultures are still living on M2 + CHX (their lifespans will be over 190 cm).

[c] All cultures (n = 18) are still living.

[d] All cultures are still living except one that stopped growing at 188 cm growth.

intrinsic heterogeneity of a given thallus appeared even more striking when observations were performed on Petri dishes. As shown in Figure 1A, besides cultures able to cover a dish and others showing growth arrest on the entire growth front, a third type was observed that exhibited signs of death on a limited area of the growth front. In all cases examined so far, the dying mycelia contained the deleted mitochondrial chromosome typical of premature death syndrome (Figure 1B; BELCOUR *et al.* 1991).

Cytoplasmic determinants and nuclear backgrounds do not play any role in the sporadic appearance of premature death. The variability was independent of the genotype of the female partner (*AS1-4 rmp+*, *AS1⁺ rmp−*, *AS1-4 rmp−*) and was also seen when both parents were *AS1-4* (data not shown).

In contrast to *AS1-4 rmp+*, the lifespan values obtained for *AS1-4 rmp−* and *AS1⁺* strains fitted well with homogeneity both at the level of each progeny (standard deviation) and between crosses (mean values). The similarity appeared even more striking when coefficients of variation were compared (SOKAL and ROHLF 1969). In fact, these values, which permit comparisons between distributions with different means, clearly showed that *AS1-4 rmp+* strains are distinct from the three other strains (Table 1). The significance of these comparisons was confirmed by the WILCOXON test (WILCOXON 1945; data not shown). This discrepancy between the *AS1-4 rmp+* strains and the other strains is emphasized when the data are plotted according to MARCOU (1961): the logarithm of the percentage of living subcultures is given with respect to growth length. Such a representation is shown in Figure 2 for the four genotypes. The curves obtained for the *AS1⁺ rmp+*, *AS1⁺ rmp−* and *AS1-4 rmp−* strains appear similar, with the exception of the plateau. This difference reveals that premature death occurs earlier than classical senescence. The sharp slope of the curves shows that, in both

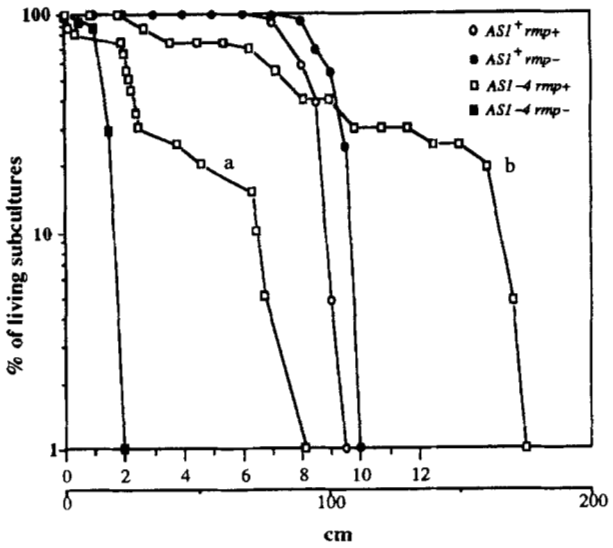

FIGURE 2.—Life-span curves of *AS1*$^+$ *rmp+*, *AS1*$^+$ *rmp−*, *AS1-4 rmp+* and *AS1-4 rmp−* grown on M2 medium. The data are reported according to MARCOU (1961) as the logarithm of the percentage of living subcultures with respect to growth length. Survivors are plotted every centimeter for *AS1*$^+$ (both strains) and *AS1-4 rmp−*, every 10 cm for *AS1-4 rmp+*. Data are taken from Table 1 (line III), except for *AS1-4 rmp+* (curve a), which was obtained from Table 1 (line IV).

TABLE 2

Premature death escape in *AS1-4 rmp−* strains grown on MR medium: the data do not fit a binomial distribution

No. of escape events per ascospore	No. of ascospores	
	Expected	Observed
0	0.698	5
1	2.784	6
2	4.993	3
3	5.307	0
4	3.702	0
5	1.771	2
6	0.588	0
7	0.134	1
8	0.020	0
9	0.002	2
10	0.000	1

Twenty *AS1-4 rmp−* ascospores were obtained from *AS1-4 rpm+* × *AS1*$^+$*rmp−* crosses. Each germinating thallus was split into 10 implants giving rise to 10 subcultures on MR medium. Each ascospore can give rise to subcultures that at least partly escape premature death (from zero to 10 subcultures, first column). The expected distribution of the 20 ascospores into these 11 classes was calculated according to a binomial distribution using the observed frequency of subcultures that escaped premature death: 57/200, *i.e.*, 0.285.

cases, there is no escape from death once the primary event has occurred. As revealed by the coefficients of variation (Table 1), the distributions are homogeneous. In contrast, the curves obtained for *AS1-4 rmp+* strains are complex. In certain samples (see Figure 2, curve a), the first plateau is lacking, as if in some *AS1-4 rmp+* cultures, death were determined as soon as ascospore germination as it is in all *AS1-4 rmp−* cultures. Regardless of this particular point, all the samples share a common feature (see Figure 2, curves a and b as examples): there are always several plateaus, as if the probability of an efficient triggering event were very low in this genetic context (see DISCUSSION).

To investigate the apparent homogeneity of the *AS1-4 rmp−* genotype, an extensive sampling was performed. Thalli obtained from 20 *AS1-4 rmp−* ascospores were recovered from germination medium and divided into 20 implants allowed to grow on Petri dishes (10 on M2 medium and 10 on MR medium). All 200 subcultures grown on M2 medium showed a typical premature death phenotype while heterogeneity appeared on MR medium. Reminiscent of the behavior of *AS1-4 rmp+* grown on M2 medium, three kinds of subcultures were observed with *AS1-4 rmp−* grown on MR medium. First, as expected, most subcultures showed the premature death phenotype all along the growth front of the thallus. Second, other subcultures were able to cover a Petri dish without showing any sign of death. Third, some subcultures exhibited two sectors: one showed premature death while the other grew to the edge of the dish. Statistical analysis of the data (Table 2) showed that the probability of premature death escape did not fit a binomial distribution, as expected from its random appearance. Rather, it depends on the original ascospore. The simplest explanation of these observations is that the ascospores differ from each other at the time of germination with respect to a component required for premature death. If premature death were expressed at a given threshold of this component, one could imagine that its concentration would decrease from the dead front to more distal parts of the thallus. This question was addressed as follows: implants were collected from *AS1-4 rmp−* cultures every 5 mm, at increasing distances from the dead front, and allowed to grow on a second Petri dish. The data clearly showed a gradient effect. Ninety percent of the implants taken at 5 mm of a dead front gave rise to cultures showing premature death. This figure progressively decreased to <10% of the explants collected >60 mm from a dead front (data not shown).

As a preliminary conclusion, several points must be noted. First, premature death syndrome (defined by its characteristic mtDNA deletion) is not limited to the *AS1-4 rmp−* genotype: it is shared by the *AS1-4 rmp+* genotype. Second, a predisposition to express this syndrome is controlled by both the *rmp* gene (the *rmp−* allele being more efficient than the *rmp+* allele) and by culture medium (M2 being a better enhancer than MR). Third, the ability to show the syndrome is an intrinsic property of each ascospore. Fourth, it is noteworthy that premature death can be limited to one part of a thallus. Although linked through anastomoses be-

TABLE 3

Effect of various drugs on growth parameters of *AS1*$^+$ *rmp*+ and *AS1-4 rmp*+ strains

Antibiotic[a]	Dose (μg/ml)	Linear growth rate (%)[b]		Lag (day)[c]	
		AS1$^+$	*AS1-4*	*AS1*$^+$	*AS1-4*
None	—	100	100	—	—
CHX	2	91–100[d]	57–92[d]	1	1.3
Pm	500	86–100[d]	65–77[d]	No	No
	750	52–76[e]	40–68[f]	1.5	5
Ksg	500	68	73	No	No
	1000	50	65	No	No
EB	10	90	90	1.5	6

[a] CHX, cycloheximidine; Pm, paromomycin; Ksg, kasugamycin; EB, ethidium bromide. The first three antibiotics were tested in M2 medium and EB in MR medium.

[b] Elongation rate of the mycelium on drug-containing medium expressed as percentage of the elongation rate on drug-free medium. 100% means 5.5 mm per day for *AS1*$^+$ and 6.5 mm per day for *AS1-4*.

[c] Lag time before the strain resumed growth after its transfer to drug-containing medium.

[d–f] In these three cases, the slope of the growth curve displayed a clear-cut change at 9 days ([d]), 11 days ([e]), 18 days ([f]). The two numbers refer to the first and the second slope.

tween filaments, two adjacent subclones can display different phenotypes (see Figure 1). This suggests that the causative agent of the syndrome is not highly invasive, at least in terms of lateral diffusion. Last, premature death may occur at a given threshold of a component, which could be the deleted molecule *per se*.

Drug effects: differences between premature death and senescence: The sporadic appearance of premature death in *AS1-4 rmp*+ strains and its sporadic disappareance in *AS1-4 rmp*− strains appeared challenging. We left aside the analysis of differences between M2 and MR media, which requires long and tedious studies. Instead, we attempted to modify the environmental or genotypic backgrounds of the *AS1-4* strains to see if the syndrome could be triggered or suppressed in a controlled fashion.

Phenotypic changes could easily be achieved with drugs. We focused mainly on antibiotics known to alter protein synthesis, since one of the nuclear partners of premature death (*AS1*) is a gene encoding a cytosolic ribosomal protein (DEQUARD-CHABLAT *et al.* 1986; DEQUARD-CHABLAT and SELLEM 1994). These antibiotics include cycloheximide, paromomycin and kasugamycin. While cycloheximide inhibits translation without altering accuracy, the two other inhibitors have opposite effects on accuracy. Paromomycin is known to increase translational errors while kasugamycin is thought to decrease them (see DISCUSSION for references). Table 3 shows the effects of these drugs on growth parameters of *AS1*$^+$ *rmp*+ and *AS1-4 rmp*+ strains (the *AS1*$^+$ *rmp*− strain gave the same data as the *AS1*$^+$ *rmp*+ strain, while the *AS1-4 rmp*− strain died too early to allow measurement of growth rates). The *AS1-4 rmp*+ strain clearly appears more sensitive to paromomycin and more resistant to kasugamycin than the wild-type strain. These properties are shared by other *AS1* alleles (COPPIN-RAYNAL 1981; KIEU-NGOC and COPPIN-RAYNAL 1988). The *AS1-4 rmp*+ strain also shows an increased sensitivity toward cycloheximide (Table 3). As shown in Table 1, the three drugs modify the lifespan of the wild-type strains. While cycloheximide was previously shown to decrease lifespan, at least on corn-meal medium (BELCOUR and BEGEL 1980), this is the first report of assays with paromomycin and kasugamycin. The first drug increases while the second decreases the lifespan of wild-type strains.

The lifespans of *AS1-4 rmp*+ were measured with and without cycloheximide. They appeared similar in the two conditions (Table 1, lines VII 1 and VII 2). To confirm this lack of effect, observations were performed on strains grown in Petri dishes: cycloheximide was unable either to trigger premature death in *AS1-4 rmp*+ strains or to alleviate it in *AS1-4 rmp*− strains, even when added to the germination medium (data not shown). The effects of paromomycin and kasugamycin on *AS1-4 rmp*+ strains were also measured in race tubes. Both clearly increased the lifespan (Table 1, compare lines VII 3 and VII 4 with line VII 1). The two antibiotics were assayed on *AS1-4 rmp*− strains grown on Petri dishes. Nearly half of the strains (30/79) were saved from premature death when allowed to grow on M2 supplemented with paromomycin. Kasugamycin was also able to promote this phenotypic rescue although to a much lower extent (three strains saved among 47 tested). When grown on drug-supplemented medium, some of the paromomycin (kasugamycin)-rescued *AS1-4 rmp*− strains showed limited signs of death while others were able to cover serial Petri dishes. The effect of paromomycin was clearly dose-dependent. Routinely, tests were performed with 500 μg/ml. The same data were observed with 400 μg/ml while the number of cultures rescued by the drug decreased at 300 μg/ml. Finally, no strains were saved below this concentration (tests performed from 50 to 250 μg/ml; data not shown). The effect of kasugamycin was assayed at a single concentration (1 mg/ml). It is noteworthy that when a *AS1-4 rmp*− strain was saved by growth on drug-supplemented medium, the premature death syndrome was clearly delayed when the strain was transferred to drug-free medium. In some cases, a large sector of the thallus was even able to cover a Petri dish. Given the size of the explant (a few cubic millimeters) with respect to that of a Petri dish (9 cm in diameter) and the volume of drug-free medium in the dish (40 ml), this striking carry-over cannot be explained simply as a drug dilution effect.

Ethidium bromide was also used that is known to increase the lifespans of wild-type strains and to rejuve-

nate senescent cultures (BELCOUR and BEGEL 1980; KOLL *et al.* 1984). As shown in Table 3, this drug had only a moderate effect on *AS1-4 rmp+* cultures. However, growth resumption of *AS1-4 rmp+* was slower than that of wild-type cultures after transfer to medium supplemented with the drug. In parallel experiments, ethidium bromide (5–10 μg/ml) was applied to arrested mycelia suffering either from senescence (genotype: $AS1^+$ *rmp−*) or from premature death (genotype: *AS1-4 rmp−*). While no mycelia (0/93) were saved from premature death, nearly half (23/62) recovered from senescence and resumed normal growth. Even when used as early as ascospore germination, ethidium bromide was unable to prevent *AS1-4 rmp−* individuals from expressing premature death syndrome. Thus, contrary to senescence, premature death is insensitive to ethidium bromide treatment.

As a preliminary conclusion, it appears that premature death and senescence respond differently to the drugs tested. Concerning premature death, only paromomycin and kasugamycin showed an effect: both antibiotics increase the lifespan of *AS1-4 rmp+* and both are able to rescue *AS1-4 rmp−* although in a sporadic manner.

Effect of additional mutations: epistasis and suppression: The *AS3-2* mutation that decreases (BELCOUR *et al.* 1991) the lifespans of wild-type strains was introduced into the *AS1-4* backgrounds. Mutations of the *AS3* gene are highly pleotropic and lead to a very short lifespan (BELCOUR *et al.* 1991). Isolation and characterization of *AS1-4 AS3-2* double mutant strains are described in MATERIALS AND METHODS and were studied on M2 medium. The results can be summarized as follows. *AS3-2 rmp+* strains exhibited their senescent front around 4 cm of growth while the seven double mutant strains recovered were able to fill a Petri dish, as observed for most of the *AS1-4 rmp+* strains. *AS3-2 rmp−* strains showed their senescent front at 8 cm of growth while the eight double mutant strains recovered displayed a heterogeneous behaviour. Some *AS1-4 AS3-2 rmp−* strains died after 2 cm of growth while some others were still alive after 18 cm. Thus, the *AS1-4* mutation is clearly epistatic upon the *AS3-2* mutation in both the *rmp+* and *rmp−* contexts. Unfortunately, too few *AS1-4 AS3-2 rmp+* strains were recovered to determine whether they could exhibit signs of death sporadically, as do some *AS1-4 rmp+* strains. However, this sporadicity was shown by the double mutant strains recovered in the *rmp−* background. The epistatic effect of the *AS1-4* mutation upon the *AS3-2* mutation is confirmed by molecular analyses of the mitochondrial genome. In fact, senescence of the *AS3* mutants is characterized by a high level of senDNAα (SAINSARD-CHANET *et al.* 1993) while the *AS1-4 AS3-2 rmp−* strains show the site-specific deletion characteristic of the premature death (data not shown).

One other nuclear mutation (*su8-1*) was introduced in the *AS1-4* backgrounds for specific reasons. The *su8-1* mutation is a dominant opal tRNA suppressor (DEBUCHY and BRYGOO 1985) used as a selective marker in some of our libraries (BRYGOO and DEBUCHY 1985; PICARD *et al.* 1991). If such a library is used to clone the *rmp* gene, it will be necessary to know the effect of the *su8-1* mutation in the *AS1-4* background. The lifespans of $AS1^+$ *su8-1* strains appear slightly (but not significantly) shorter than those of the wild type (P. SILAR, personal communication). However, the *AS1-4 su8-1* strains (either *rmp+* or *rmp−*) exhibit the premature death phenotype (tests performed on M2 medium). As an example, the 13 *AS1-4 su8-1 rmp+* strains (recovered from a $AS1^+$*su8-1 rmp−* × *AS1-4* $su8^+$ *rmp+* cross) all stopped growing before 2.5 cm, while the 17 *AS1-4* $su8^+$ *rmp+* strains (obtained from the same cross) all cover a Petri dish, with the exception of three strains showing limited signs of death. All *AS1-4 su8-1 rmp−* strains displayed a worsened premature death phenotype: the dead front occurred earlier in *AS1-4 rmp− su8-1* strains than in *AS1-4 rmp−* $su8^+$ strains. In all the *AS1-4 su8-1* strains examined, mycelial death was found associated with the mtDNA site-specific deletion (data not shown). The fact that the *AS1-4 rmp+ su8-1* strains display the phenotype of *AS1-4 rmp−* strains (systematic premature death) is easily explained if the *rmp+* allele carries a nonsense (UGA) mutation. This hypothesis leads to two simple predictions. First, *rmp+* should be recessive with respect to its *rmp−* allele and second, the *su8-1* mutation should be dominant concerning premature death, as it is when tested upon other markers.

Tests for dominance/recessiveness of the *AS1*, *rmp* and *su8* alleles with respect to premature death: To perform the most reliable test, forced vegetative heterocaryons were used. One or the other of two auxotrophic markers were introduced, through crosses, in the four relevant genotypes and forced heterocaryons were obtained in all possible combinations (MATERIALS AND METHODS). The results, shown in Table 4, demonstrate that *rmp−* is dominant over its *rmp+* allele: the *AS1-4 rmp+* mycelium is unable to rescue its *AS1-4 rmp−* partner although a true heterocaryotic state is achieved, as demonstrated by growth on minimal medium before death. In the two cases examined (two assays each), the mycelial death was associated with the specific mitochondrial deletion that characterizes this syndrome (data not shown). Data from Table 4 also provide evidence that the *AS1-4* mutant is recessive whatever the *rmp* background. In fact, a *AS1-4 rmp−* mycelium is completely rescued by a $AS1^+$ partner, even if the latter also carries the *rmp−* information. This conclusion was recently strengthened following the cloning of the $AS1^+$ gene. *AS1-4 rmp+* strains carrying a transgenic $AS1^+$ gene (integrated ectopically) were crossed with a *AS1-4 rmp−* strain: the *AS1-4 rmp−* progeny never displayed the premature death phenotype when they carried the $AS1^+$ transgenic information (DEQUARD-CHABLAT and SELLEM 1994).

TABLE 4

Forced vegetative heterocaryons between strains carrying the mutant or wild-type *AS1* alleles and the *rmp+* or *rmp−* alleles

	leu1-1 strains			
lys2-1 strains	*AS1-4 rmp−*	*AS1-4 rmp+*	*AS1⁺ rmp−*	*AS1⁺ rmp+*
AS1-4 rmp−	NT	PD (1)[a]	L (2)	NT
AS1-4 rmp+	PD (8 + 1)[b]	L (3)	L (9 + 1)[c]	L (4)
AS1⁺ rmp−	L (18)	L (7)	NT	NT
AS1⁺ rmp+	L (11)	L (3)	NT	NT

NT, not tested; PD, premature death; L, normal lifespan. Numbers in parentheses refer to heterocaryons independently obtained (MATERIALS AND METHODS).

[a] Two additional heterocaryons did not resume growth when tested for lifespan.

[b] Eight heterocaryons clearly showed the premature death phenotype and one grew a little more before death. Two additional heterocaryons did not resume growth when tested for their life span.

[c] One heterocaryon among 10 showed limited signs of death but continued growth on the major part of the thallus.

The dominance/recessiveness of the *su8-1* mutation could not be tested by the heterocaryon-based test since the *su8-1* mutation suppresses one of the two auxotrophic markers available to obtain forced vegetative heterocaryons. Therefore, we took advantage of the cloning of this gene (DEBUCHY and BRYGOO 1985). In fact, the cloned *su8-1* mutant gene has been used as a selective marker in transformation experiments (DEBUCHY and BRYGOO 1985; PICARD *et al.* 1991) showing that this suppressor is dominant, as usually observed for tRNA suppressors. For our purpose, the *su8-1* mutation was introduced by transformation into a *AS1⁺ rmp+ su8⁺* strain (see MATERIALS AND METHODS). Several primary transformants were crossed with a *AS1-4 rmp− su8⁺* tester strain. Among the offspring, 21 *AS1-4 rmp− su8⁺* and 30 *AS1-4 rmp+ su8⁺* strains carrying the *su8-1* transgenic marker were recovered. All showed premature death. Thus the *su8-1* mutation is dominant with respect to this syndrome: its effect is the same in the presence and absence of the *su8⁺* allele. This strengthens the idea that *rmp+* carries a nonsense (UGA) mutation.

The *mat* information does not encompass the *rmp* gene: During the numerous crosses performed between the *AS1-4 rmp+* (*mat+*) or *rmp−* (*mat−*) and the wild-type strains, recombination between *rmp* and *mat* was never observed. These data left open an important question: is the *rmp* gene part of the mating-type information or is it a gene tightly linked to the *mat* locus? We addressed this question in three sucessive steps. First, we asked whether the *rmp+ mat+* and *rmp− mat−* relationships were maintained in other wild-type strains of *P. anserina*. Second, we took advantage of the cloning of the *mat−* genes and of the dominance of the *rmp−* allele to introduce the *mat−* information into a *AS1-4 rmp+* (*mat+*) strain. Finally, we tried to directly obtain recombination between the *rmp* and *mat* genes, using an ascospore marker tightly linked to the *mat* locus.

A collection of *P. anserina* strains isolated from different geographical locations (geographical races) was established by RIZET (1952 and personal communication). Most of these strains differ from our reference strain (S) and from one another at least by nuclear genes responsible for vegetative incompatibility (RIZET and ESSER 1953; BERNET 1965). Furthermore, their mitochondrial genomes also display differences, especially in the presence or absence of optional sequences (CUMMINGS *et al.* 1990; L. BELCOUR, unpublished results). To learn the status of the mating-type haplotypes of these strains with respect to the *rmp* gene, it was necessary to join these mating types and the *AS1-4* mutation carried by the S strain. The experiments could obviously be performed only with races able to give fertile crosses with the S strain, namely the A, M, N, s, T, U, W, and Z races. It was shown that the T strain in fact belongs to another close species, *P. comata*, despite its low fertility with the *P. anserina* S strain (J. BEGUERET, personal communication). Successive back-crosses permitted us to obtain the two nuclear genotypes (*mat+* and *mat−*) required for each strain in two different cytoplasmic backgrounds, taking into account that mitochondria are transferred from the maternal parent through sexual crosses in *P. anserina* (BELCOUR and BEGEL 1977). For example, the two mating-type haplotypes of the A race were associated with the *AS1-4* mutation in the two possible mitochondrial contexts, (A) and (S) (see MATERIALS AND METHODS). As for the T (*P. comata*) race, the experiments were performed only in the cytoplasmic S background since the cross is fertile only in one direction (female S × male T). The data (not shown) permitted the conclusion that the two mating-type haplotypes of six strains (A, M, T, U, W, and Z) carried the same *rmp* allele, namely the *rmp−* allele. The *AS1-4* mutation led to premature death syndrome (with the specific mitochondrial deletion) whatever the mating-type information tested (*mat+* or *mat−*). Thus only the N, s and S races bear the *rmp+* allele, linked to the *mat+* information. These observations lead to

TABLE 5

The *mat*− specific DNA sequence does not contain the *rmp*− allele

Type of strain	Genotype: Resident markers	Genotype: Transgenic markers	Normal life span (nb/total)
A	*AS1-4*, Δ*mat*+ (*rmp*+ ?)[a]	None	20/20
B	*AS1-4*, Δ*mat*+ (*rmp*+ ?)[a]	*mat*− (*rmp*− ?)[a]	29/29
C	*AS1-4*, *mat*+ *rmp*+	None	36/39[b]
D	*AS1-4*, *mat*+ *rmp*+	*mat*− (*rmp*− ?)[a]	15/17[b]

A cross was performed between a *AS1-4 mat*+ *rmp*+ strain and a *AS1*$^+$ Δ*mat*+ transformant strain carrying the transgenic *mat*− information: the *AS1-4* progeny was examined for genotype and lifespan.

[a] The *rmp* in parentheses indicates that before the analysis of this progeny, we did not know either if the *rmp*+ gene was deleted along with the *mat*+ information or if the plasmid *mat*− DNA brought along the *rmp*− gene.

[b] The remaining strains did not show premature death, but they displayed a slight sign of death on a small part of the front although continuing growth, as did the others, on the major part of the thallus.

three conclusions. First, the *rmp*+ allele appears to be the less frequent in nature (three cases among 18). Second, the mitochondrial deletion occurs in all the cytoplasmic backgrounds tested. Third, it appears unlikely that the *rmp* gene can be an intrinsic part of the *mat* information.

A second, more direct, approach became possible when the *P. anserina mat* genes were localized to small DNA fragments: 3.8-kb for *mat*+ and 4.5-kb for *mat*− (DEBUCHY and COPPIN 1992). During studies of the mating types of *P. anserina*, a strain deleted of the *mat*+ information was recovered and a plasmid carrying the *mat*− information was introduced into this Δ *mat* strain. The transgenic, ectopically integrated *mat*− information was fully expressed, allowing the strain to be crossed with any *mat*+ strain (COPPIN *et al.* 1993). For our purpose, it was crossed with a *AS1-4 rmp*+ *mat*+ strain. As shown in Table 5, the transgenic *mat*− DNA, which brings all the *mat*− functions to the Δ *mat* recipient, does not carry the *rmp*− gene. The three new kinds of strains (Table 5: A, B, D) behave like the control *AS1-4 rmp*+ *mat*+ (C): they did not show premature death phenotype. If the *rmp*− gene were part of the *mat*− information, it should be expressed in the B and D strains, because it was shown to be dominant over the *rmp*+ allele (see above).

These data prompted us to carry out a large-scale search for recombination between the *rmp* and *mat* genes. This appeared challenging: as shown by MARCOU *et al.* (1979), besides the well-known high chiasma interference observed between the centromere and the *mat* locus, there is a preferential localization of meiotic exchanges, the *mat* locus lying in a very low recombination frequency region. However, Y. BRYGOO (personal communication) used a rationale that aided him to map several genes tightly linked to *mat*. While double crossover frequency is very low in the total population of asci, it appears strongly increased among the minority population of asci showing FDS for a marker mapping far away from the centromere (*i.e.*, *mat* or a gene tightly linked to it). This results in an increased probability of finding a meiotic exchange in the *mat* region. Therefore, we used the recessive *PmA-1* mutation that maps <0.5 cM from the *mat* locus on its distal side (Y. BRYGOO, personal communication). Because *PmA-1* leads to small-sized spores, FDS asci for this marker (which contain two large and two small ascospores) can be scored by eye. A *AS1-4 rmp*+ *mat*+ *PmA*$^+$ × *AS1*$^+$ *rmp*− *mat*− *PmA-1* cross was performed: 174 SDS and 200 FDS events for *PmA* were analyzed for the segregation patterns of *AS1* and *mat* (Table 6). The *AS1-4* strains were then tested for premature growth arrest. Unfortunately the issue might be confused, leading to misinterpretation, mainly because of the sporadic expression of the premature death phenotype among *AS1-4 rmp*+ strains (see above). The difficult and time-consuming work required in further analysis of many false candidates led us to discard those FDS asci of which the two *AS1-4* ascospores did not display the same phenotype (either premature death or normal lifespan). Fourteen asci were further analyzed: their ascospores were backcrossed to either a wild-type or a *AS1-4* strain and progeny tested for the putative new associations between *mat* and *rmp*. Twelve were false candidates but two confirmed a recombination event between *rmp* and *mat*. Both belonged to the triple FDS class of Table 6; analysis of one of them clearly showed that these asci showed a SDS for *rmp* while displaying a joint FDS for all three other markers. This could mean that *rmp* is the more distal gene. If *rmp* were distal to *PmA*, a recombination event between *mat* and *PmA* should entail recombination between *mat* and *rmp*. The six asci showing recombination between *mat* and *PmA* (Table 6) were carefully reexamined. They did not display the phenotype expected of asci also recombinant for *rmp* and *mat*. Furthermore, *AS1-4* ascospores from three of them were backcrossed to wild type: the progeny showed parental associations for *rmp* and *mat*. Therefore, *rmp* is not distal to *PmA*. The most likely position for *rmp* is proximal to *mat*. The two recombinant asci are thus explained by

TABLE 6

Analysis of asci from the *AS1-4 rmp+ mat+ PmA*$^+$ × *AS1*$^+$ *rmp− mat− PmA-1* cross

Genetic map Intervals	c——— *AS1* ——— *mat* ——— *PmA*——— I	II	III				
	Segregation				Minimal no. of crossovers in interval		
Selected asci[a]	*AS1*	*mat*	*PmA*	No. of asci	I	II	III
	SDS	SDS	SDS	61	1	0	0
	FDS	SDS	SDS	107	0	1	0
A (174)	FDS	FDS	SDS	2	0	0	1
	SDS	SDS	SDS	3	1	1	0
	FDS	SDS	SDS	1	0	1	1
	FDS	FDS	FDS	161[b]	0	0	0
	SDS	FDS	FDS	29	1	1	0
B (200)	FDS	FDS	FDS	7	0	2	0
	FDS	SDS	FDS	2	0	1	1
	SDS	SDS	FDS	1	1	0	1

The upper line gives the relative order of the three markers along the right arm of LGI (c, centromere). The distances are not to scale.

[a] The recessive *PmA-1* mutation decreases the size of the ascospores. Thus SDS for *PmA* gives rise to asci with four normal-sized spores (A) while FDS leads to asci with two normal-sized and two small spores (B). Due to the low level of *PmA* FDS and the low level of five spored asci, *PmA* FDS asci analyzed were mostly four-spored. On the contrary, analysis of *PmA* SDS progeny was systematically performed with five-spored asci: three dicaryotic spores (normal size) and two monocaryotic spores (one normal size, one small size). Number of asci in parentheses.

[b] Recombination between *mat* and *rmp* was observed in two of these 161 asci.

two crossovers: one between *AS1-4* and *rmp* and the other between *rmp* and *mat*. This is not really unusual: a crossover near the *mat* locus is frequently accompanied by a second in either of the two proximal intervals (see crossovers in interval III of Table 6). This tentative mapping might help in cloning the *rmp* gene by chromosome walking from the cloned *mat* locus.

Last but not least, this study provided the opportunity to observe the effect of the recombinant association *rmp− mat+* upon the lifespan of a wild-type (*AS1*$^+$) strain. It has been shown by RIZET (1953) that the lifespans of our reference strain (measured on MR medium) differ depending on mating type: the process of senescence is delayed in *mat+* strains compared with *mat−* ones. However the differences, although significant, are not so striking as the differences between *AS1-4 rmp+ mat+* and *AS1-4 rmp− mat−* lifespans (BELCOUR *et al.* 1991; this paper, Table 1 lines I and II). Deletion of the *mat* locus and reconstruction of transgenic *mat+* and *mat−* strains previously showed that the gene acting in the wild-type strains was not part of the *mat* information (COPPIN *et al.* 1993). In the course of this study, we observed the following mean lifespans mesured on corn meal medium (MR): 33 ± 9 cm, 16 ± 2 cm, 17 ± 3 cm for *AS1*$^+$ *rmp+ mat+*, *AS1*$^+$ *rmp− mat−* and *AS1*$^+$ *rmp− mat+*, respectively. The *rmp+ mat−* combination could not be tested because of the presence of the *PmA-1* mutation that modifies lifespan (data not shown). However, it is noteworthy that the *rmp− mat+* and *rmp− mat−* genotypes lead to identical lifespans.

DISCUSSION

Innocuity of the mating-type genes in premature death syndrome: In *N. crassa*, the mating-type genes have been implicated in the process of vegetative incompatibility since its discovery (BEADLE and COONRADT 1944). Similarly, in *P. anserina*, since the discovery of the senescence phenomenon (and long before its explanation by mtDNA rearrangements), the mating-type genes or genes tightly linked to them were proposed to play a role in the process (RIZET 1953; MARCOU 1961). More recently, the mating-type region has also been implicated in premature death (BELCOUR *et al.* 1991). Cloning and sequencing of the mating-type genes in both fungi have solved the problem with opposite outcomes. In *N. crassa*, the *mtA1* and *mta1* genes are clearly bifunctional: they are involved in both sexual reproduction and vegetative incompatibility (GLASS *et al.* 1990; STABEN and YANOFSKY 1990). In *P. anserina* it was shown that the mating-type information has no role in the timing of senescence (COPPIN *et al.* 1993). In this paper, we also show, through three successive approaches, its innocuity in premature death.

In a first step, we used seven geographical races of *P. anserina* and one other species, *P. comata*, able to cross (more or less efficiently) with our reference strain. Most displayed the premature death syndrome in the presence of the *AS1-4* mutation, whatever their mating type. We then took advantage of the cloning of the *mat* genes (PICARD *et al.* 1991). If the *rmp* gene were part of the *mat* information, a *AS1-4 mat+ mat−** transgenic

strain (in which *mat*− * is the transgenic information) should display premature death because, as shown in this paper, the *rmp* allele linked to *mat*− (*rmp*−) is dominant upon the *rmp*+ allele (linked to *mat*+). On the contrary, the transgenic strain still displayed its original *AS1-4 mat*+ (*rmp*+) phenotype. To avoid any effect of the *mat*+ information upon the expression of the *mat*− genes (introduced into the same nucleus), we used also a strain deleted of *mat*+ information (COPPIN *et al.* 1993). The results remained the same. Thus the *mat*− information does not encompass the *rmp*− gene. In a third step, we definitively showed that this gene is outside the mating-type locus by recombination experiments. Furthermore, although recombination between the *rmp* and *mat* genes was observed in only two asci, the data as a whole suggest that the *rmp* gene lies proximal to the *mat* locus with respect to the centromere.

Does the *rmp* gene also control the timing of the senescence process? It is noteworthy that, in our reference strain, the *mat* haplotypes control the timing of both senescence and premature death. Thus the two syndromes may be under the control of a single gene (*rmp*) or there may be two genes: the *rmp* gene involved in premature death and another controlling the timing of senescence. The only arguments that can clarify this problem are indirect. Recombination between the *rmp* gene and the *mat* locus did not separate the two putative genes. However, only two recombinant asci have been obtained. Analysis of the geographical races could provide complementary information. It was not difficult to determine the status of the *mat* haplotypes of these races with respect to premature death since this assay required only the association of each haplotype with the *AS1-4* mutation. Understanding their status with respect to senescence is far more complicated. It is indeed well known that many nuclear genes modify the timing of senescence (GRIFFITHS 1992 for a review; BELCOUR *et al.* 1991; P. SILAR, personal communication). Therefore, each of the *mat* haplotypes of a given race must be introgressed into our reference strain (S). Reliable data are available only for the s race (considered to be closely related to the S race) and for the *P. comata* species (which was submitted to the above procedure). It appears that the S and s races exhibit the same *mat* haplotype features, *mat*+ delaying the expression of both syndromes with respect to the *mat*− timing (on MR medium). In opposition, the two *P. comata mat* haplotypes, both containing the *rmp*− allele (this paper), show the lifespan of a *mat*+ (*rmp*+) S strain (M. PICARD, unpublished results) in the $AS1^+$ background. This latter fact supports a two-gene hypothesis while the other data do not disprove it. We therefore favor the two-gene hypothesis with the *rmp* gene being specifically involved in the timing of premature death. Such apparent clustering of genes involved in similar functions is a common feature of the *P. anserina* genome, probably due to strong chiasma interference and preferential localization of meiotic exchanges (RIZET and ENGELMANN 1949; KUENEN 1962; MARCOU *et al.* 1980; J. M. SIMONET, D. MARCOU and M. PICARD, unpublished results).

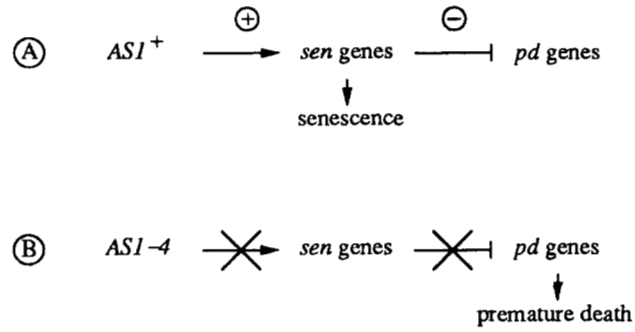

FIGURE 3.—Senescence and premature death are mutually exclusive syndromes: a formal model. (A) When the *sen* genes are expressed, senescence can occur but premature death cannot because of the repression of (at least) one of the *pd* genes. (B) In the *AS1-4* background, the *sen* genes are expressed at a too low level to trigger senescence while the *pd* genes are switched on.

Premature death and senescence are two mutually exclusive syndromes: It would be presumptuous and beyond the scope of this paper to attempt an extensive comparison between premature death and senescence. In fact, premature death has begun to be investigated recently while senescence has long been studied in several laboratories (GRIFFITHS 1992 for a review). However, because wild-type strains and their typical senescence process have been used as controls in this study, one cannot avoid noting a few points. The two syndromes resemble one other in that both are defined as cell death probably caused by mtDNA rearrangements. However, the rearrangements differ in the two situations. In the case of senescence, short mtDNA sequences (senDNAs) are amplified as circular multimeric molecules (DUJON and BELCOUR 1989 for a review). In the case of premature death, a site-specific deletion of the mitochondrial genome is observed (BELCOUR *et al.* 1991). The two syndromes also differ in other aspects. First, senescence has been found in most (if not all) genetic backgrounds while premature death has been seen only in strains carrying particular mutations of the *AS1* gene. Furthermore, as shown in this paper, the two processes are not sensitive to the same environmental conditions. Last but not least, the *AS3-2* mutation, which in the $AS1^+$ background leads to a very short lifespan with a massive accumulation of senDNAα (SAINSARD-CHANET *et al.* 1993), is hypostatic with respect to *AS1-4* since the double mutant strains display the site-specific deletion.

At the present time, only simple and formal models can be proposed to explain the above observations. One possibility is shown in Figure 3. In the $AS1^+$ background, genes that eventually lead to accumulation of senDNAs (*sen* genes) are expressed. In this context, one or several genes required for the appearance of

premature death (*pd* genes) are repressed. In the *AS1-4* background, due to translational defects, the *sen* genes are expressed at a level too low to trigger senescence. This inhibitory effect also relieves the repression of the *pd* genes, leading to premature death. This model (and others as well) raise several problems concerning the effects of the *AS1-4* mutation and the nature of the genes that trigger either senescence or premature death. The *AS1-4* mutation should impair a translational step, most probably initiation, that is critical for some messengers. As for the *sen* and *pd* genes, they should be involved (directly or indirectly) in mtDNA metabolism.

Premature death and the *rmp* gene: One prediction of the above model (Figure 3) is that conditions antagonizing the effects of the *AS1-4* mutation would lead to the appearance of senescence instead of premature death. As shown in this paper, when premature death is delayed, the strains do not show senescence. For this reason, we favor the idea that the primary targets of the conditions we have used are the *pd* genes, of which the *rmp* gene could be the paradigm. Premature death is very likely due to the accumulation of the deleted mtDNA molecule to the detriment of the wild-type molecule, up to a threshold that no longer permits cell survival. This deletion is most probably caused by an intramolecular recombination event between two copies of the α intron, namely the resident and one ectopic copy of this mobile intron (SELLEM *et al.* 1993). A striking point is that the deleted molecule is present in the wild-type ($AS1^+$) strains in trace amounts that can only be seen by PCR experiments (C. SELLEM, personal communication). Thus, three parameters may be involved in the accumulation of this molecule in *AS1-4* strains: the rate of ectopic transposition of the α intron, the rate of recombination, and the relative transmission (selection) of wild-type and deleted molecules in heteroplasmons.

Presently, because the *rmp* gene (which is the only *pd* gene identified) has not yet been cloned, the role played by the RMP protein (and the other PD proteins) is unknown. However, one can propose a simple model concerning the expression of these genes that takes into account the reported data. This model is built on three propositions. First, the *rmp*− and *rmp*+ alleles are, respectively, a functional and a mutant (UGA) form of the *rmp* gene. Second, the sporadic appearance of premature death in *AS1-4 rmp*+ strains may be due to natural suppression (readthrough) of the UGA mutant codon. Third, escape from premature death in the *AS1-4* strains can be explained by an inefficient expression of one of the *pd* genes.

The *rmp*+ allele is a mutant form of the *rmp* gene: This assumption relies on two main observations: the recessivity of the *rmp*+ allele and the effect of UGA tRNA suppressors. The recessivity of the *rmp*+ allele was established by balanced vegetative heterocaryons. Recessivity suggests a loss of function. This is easily explained if the *rmp*+ allele carries a UGA nonsense mutation as suggested by suppression data. A *bona fide* premature death syndrome was systematically triggered in *AS1-4 rmp*+ strains when they bore the *su8-1* suppressor demonstrated to be UGA tRNA suppressor (DEBUCHY and BRYGOO 1985). Although translational side effects of these mutations cannot be excluded, the most simple explanation is that the *rmp*+ allele carries a UGA nonsense mutation. Due to the position of the UGA stop codon, the RMP mutant protein could be either nonfunctional or unstable.

Sporadic appearance of premature death in *AS1-4 rmp*+ strains may be due to readthrough of the *rmp*+ UGA codon: Natural suppression (readthrough) of nonsense codons is a well-known process, especially documented for some viruses in which it is required for synthesis of proteins needed in low amounts (ATKINS *et al.* 1990 for a review). Readthrough can also cause the leakiness of some nonsense mutations. Clearly, natural suppression depends both on the nucleotide context of the codon and on the minimal level of the full-length functional protein required for the relevant function (see, as examples, KOPCZYNSKI *et al.* 1992; FEARON *et al.* 1994).

The sporadic appearance of premature death in *AS1-4 rmp*+ cultures means that, from time to time, this genotype can show the *AS1-4 rmp*− phenotype. A likely hypothesis is that readthrough of the *rmp*+ UGA codon can occur, leading to a stochastic production of the functional RMP protein that only rarely reaches the threshold required to trigger premature death. If efficient phenotypic suppression occurs very early, at the time of ascospore germination, premature death could be triggered in the whole thallus, leading to a *bona fide* syndrome. Efficient suppression later, in the growing mycelium, would result in a local production of the functional RMP protein and to a clonal appearance of premature death: only a sector of the thallus would show the syndrome. This hypothesis fits well with our analysis of the *AS1-4 rmp*+ lifespan. In fact, comparisons of the coefficients of variation obtained from lifespan data of the four genotypes studied in this paper have shown that *AS1-4 rmp*+ is distinct from the three others. Clearly, senescence ($AS1^+$ *rmp*+ and $AS1^+$ *rmp*−) and the *bona fide* premature death (*AS1-4 rmp*−) can be explained by a single efficient triggering event leading to the accumulation of either senDNAs or the mtDNA site-specific deletion. In opposition, the complex *AS1-4 rmp*+ lifespan curves suggest that, in this case, the triggering event can occur several times in the growing mycelium, each time with limited efficiency. Moreover, this hypothesis explains another feature of the *AS1-4 rmp*+ cultures, namely their heterogeneous mycelial phenotype. In fact, these thalli display sectors with more pigmentation, less aerial hyphae and fewer female organs than the surrounding areas. Attempts to maintain these states either through vegetative subcloning or

through sexual reproduction always failed, as if they were epigenetic states (data not shown). These sectors may reveal the stochastic production of the RMP protein (through suppression) at levels below the threshold required for premature death but sufficient to disturb metabolism and development.

Escape from premature death may be due to inefficient expression of a *pd* gene: The general model (Figure 3) relies on the assumption that derepression (or overexpression) of one or several genes (*pd* genes) leads to premature death. The consistent prediction is that conditions decreasing the expression of any *pd* gene should delay or abolish the appearance of the syndrome. Such conditions should affect both the *AS1-4 rmp+* and *AS1-4 rmp−* strains. It is noteworthy that culture conditions leading to premature death escape in *AS1-4 rmp−* cultures also cause the disappearance of the syndrome in *AS1-4 rmp+* cultures. These conditions are MR (corn meal extract) medium and M2 (minimal synthetic) medium supplemented with either paromomycin or kasugamycin. We do not know in which way the MR medium (compared to M2 medium) may modify gene expression in the cytosolic and (or) the mitochondrial compartments. However, life spans of wild-type and mutant strains are clearly increased on this medium (SILAR and PICARD 1994; this paper). As far as premature death is concerned, paromomycin and kasugamycin show the same effects. In presence of these drugs, *AS1-4 rmp+* has never shown any sign of death while *AS1-4 rmp−* can escape premature death.

The most likely explanation of these data is that these translational inhibitors lead to a significant decrease in the synthesis of one or several PD proteins. There are two main ways to selectively slow down protein production: decrease in initiation rates and processivity losses during elongation. Because initiation is a critical step relying on specific features of the mRNAs (KOZAK 1991; ALTMANN and TRACHSEL 1993 for reviews), its sensitivity toward antibiotics may differ between messengers. Unfortunately, the possible effects of the two relevant drugs upon initiation have not been documented in eucaryotes. However, both alter translational accuracy and thus may lead to processivity losses whose extent varies from one polypeptide to the other (see below). Paromomycin has been shown to increase translational misreading in the cytosolic and mitochondrial compartments, at least in *S. cerevisiae* (PALMER *et al.* 1979; SINGH *et al.* 1979; DUJARDIN *et al.* 1984). Phenotypic suppression of nuclear mutations by paromomycin has also been reported in *P. anserina* (COPPIN-RAYNAL 1981). Kasugamycin acts in the opposite sense, increasing accuracy, at least in bacteria (VAN BUL *et al.* 1984). Indirect evidence suggests that it may act similarly in the cytosolic compartment of *P. anserina* (KIEU-NGOC and COPPIN-RAYNAL 1988). However, to our knowledge, there is no report of a possible effect of kasugamycin in the mitochondrial compartment. Although we cannot exclude an action of these drugs on the mitochondria, it appears more sensible to explain their effect upon premature death via the cytosolic compartment.

Accuracy *per se* cannot be involved in the effect of the drugs since they act similarly upon premature death, while exerting opposite effects on accuracy. Interestingly, both increase and decrease in translational errors leads to the same consequence, namely a loss of processivity because of the failure to complete the synthesis of a full-length version of a protein. In fact, increasing and decreasing accuracy modify the tRNA-mRNA-ribosome interactions such that, in both cases, the probability of a peptidyl-tRNA dropping off the ribosome-mRNA complex during translation is increased (CAPLAN and MENNINGER 1984; KURLAND 1992 and references therein for a review). Although these aspects of translation have not been studied in eucaryotes, one can easily assume that they also play a key role in the efficiency of gene expression in these organisms. It is thus tempting to imagine that one of the PD proteins is long enough to be highly sensitive to such processivity losses. Paromomycin and kasugamycin would enhance drop-off, decreasing the rate of the relevant full-length protein below the threshold required for premature death expression.

Premature death and other related syndromes: There are two main aspects of syndromes related to mtDNA deletions: the primary events that cause deletions and the environmental and genetic parameters that lead to a preferential transmission of the deleted molecules at the expense of the wild-type genomes. In many cases, the primary event is likely an intramolecular recombination event between direct repeats. These recombination targets can be natural repeats, as documented in humans (SCHON *et al.* 1989; MITA *et al.* 1990) and in *N. crassa* (ALMASAN and MISHRA 1991). The repeats can also be created by a transposition event as demonstrated in *P. anserina* (SELLEM *et al.* 1993). Recently, PCR experiments have revealed that such deletions can occur constitutively at very low levels in several organisms such as humans (CHEN *et al.* 1995; MELOV *et al.* 1995), *Caenorhabditis elegans* (MELOV *et al.* 1994) or *P. anserina* (C. SELLEM, personal communication). However, diseases appear only if the level of deleted mtDNA molecules increases and becomes the major part of the mitochondrial genomes in the relevant tissues or organisms. This can be explained by a continual high-level production of the deleted molecules from the wild-type genome and/or by a preferential transmission of the deleted genome through cell divisions. The role played by these two parameters in the accumulation of deleted mtDNAs remains to be understood in most organisms.

Although the controls of mtDNA integrity and transmission have been extensively studied in *S. cerevisiae,* there is as yet no explanation for the spontaneous high-level production of *rho*$^-$ (mtDNA deletions) or for the role played by mitochondrial protein synthesis in the maintenance of mtDNA integrity in this yeast (EPHRUSSI

1953; FAYE *et al.* 1973; MYERS *et al.* 1985). The recent discovery that in other yeasts, in which *rho*$^-$ mutations had never been observed, *rho*$^-$ production can occur in particular nuclear backgrounds may clarify this problem (HAFFTER and FOX 1992; CHEN and CLARK-WALKER 1993; CHEN *et al.* 1995). In addition, the parameters that control the relative transmission of *rho*$^-$ and *rho*$^+$ genomes have also been investigated in *S. cerevisiae.* Clearly this relative transmission relies on both the structure of the relevant *rho*$^-$ genome and the genetic background. In fact, in the wild-type nuclear context, the preferential transmission of certain *rho*$^-$ variants (called hypersuppressives) has been explained by a structural replication advantage of these molecules with regard to the *rho*$^+$ genome (BLANC and DUJON 1980; DE ZAMAROCZY *et al.* 1981). However, recent studies have emphasized the role played by nuclear genes in these biased transmissions (ZWEIFEL and FANGMAN 1991; LOCKSHON *et al.* 1995). These studies and those performed in other eucaryotes easily amenable to genetic dissection will help to understand similar problems encountered in more complex systems.

Among models alternative to yeasts, filamentous fungi occupy an interesting position for two reasons. First, they are obligate aerobes and second, they can show degenerative processes leading to death that are linked to major rearrangements of the mitochondrial genome (see GRIFFITHS 1992 for a review). With respect to human diseases resulting from mtDNA deletions, data from *N. crassa* are especially striking. In *N. crassa,* a nuclear recessive mutation of the *nd* (natural death) gene causes cell death through deletions of the mtDNA (SEIDEL-ROGOL *et al.* 1989). These deletions are probably due to hyperactivate recombination events between direct repeats. Thus, the *nd* gene has been assumed to play a key role in mtDNA metabolism (BERTRAND *et al.* 1993). The *P. anserina* and *N. crassa* situations are highly reminiscent of those found in humans. In fact, human patients suffering from a sporadic (spontaneous) disease show a single mtDNA deletion while in diseases associated with a nuclear mutation the affected individuals harbor multiple mtDNA deletions (see WALLACE 1992; POULTON 1992; SCHAPIRA 1993 for reviews). In all cases, the mechanisms that underlie the expansion of the deleted molecules are still debated. Cloning of the *P. anserina rmp* and *N. crassa nd* genes, along with the identification of other genes involved in the two fungal syndromes, may shed light on the relevant human diseases. Indeed, one can assume that, although the problems are far more complex in multicellular organisms than in fungi, the basic controls of mtDNA integrity and transmission have been conserved through evolution.

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