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Genetics

A site-specific deletion in mitochondrial DNA of *Podospora* is under the control of nuclear genes

(translational accuracy/senescence/intron/fungi/myopathy)

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ABSTRACT **In the filamentous fungus *Podospora anserina*, the association of two nuclear genes inevitably leads to a "premature death" phenotype consisting of an early end of vegetative growth a few days after ascospore germination. Mycelia showing this phenotype contain a mitochondrial chromosome that always bears the same deletion. One of the break points is exactly at the 5′ splice site of a particular mitochondrial intron, suggesting that the deletion event could result from molecular mechanisms also involved in intron mobility. One of the nuclear genes involved in triggering this site-specific event belongs to the mating-type minus haplotype; the other is a mutant allele of a gene encoding a cytosolic ribosomal protein.**

In obligate aerobes, rearrangements of mitochondrial DNA (mtDNA) are responsible for various deleterious symptoms, such as maternally inherited male sterility in plants (1), several mycelial degenerative phenomena in fungi (2), and, as described more recently in humans, numerous neuromuscular (3) or hematological (4) diseases. In some cases, nuclear genes have been shown to control the mtDNA rearrangements (5, 6). The molecular mechanisms producing these rearrangements and the role played by nuclear-encoded proteins remain unclear in all cases.

In the filamentous fungus *Podospora anserina*, vegetative growth is limited by a maternally inherited syndrome, called senescence, ending in cessation of mycelial elongation and apical cell death (7, 8). In this species, senescence is clearly associated with mtDNA rearrangements and most probably is caused by them. More precisely, it has been shown that short mtDNA sequences are amplified as circular multimeric DNA molecules in senescent cultures (9–14). It was shown that the most frequently amplified sequence corresponds exactly to a mitochondrial intron (15), intron α, and that most of the mutations allowing mycelia to escape senescence are rearrangements in intron α (12, 16–18). It has been known for a long time (19) that the nuclear genome controls the life-span of the fungus. For instance, the life-span of our reference strains differs according to their mating type: the process of senescence is delayed in *mat*+ strains compared with *mat*− ones. However the differences, although significant, are not striking (see Table 1). Moreover the functions encoded by these genes are still unknown.

The work presented here follows the observation that, in *Podospora*, mutations in several genes involved in the control of translational accuracy have an effect on the life-span (A. Raynal, personal communication; Table 1). As a first step, we focused our attention on the *AS*1–4 mutation. This particular allele confers a very long life-span to *mat*+ mycelia, while, when associated with *mat*−, it leads systematically to the premature death phenotype, consisting of an early end of mycelial growth a few days after ascospore germination.

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We showed that mycelia presenting this phenotype were in all cases heteroplasmic and that they systematically contained the same deleted chromosome in addition to the wild-type mitochondrial chromosome. A remarkable observation is that one of the boundaries of the deletion is exactly at the 5′ splice site of intron α. This suggests that the deletion event could result from molecular mechanisms also involved in intron mobility. One of the nuclear genes involved in triggering this site-specific recombinational event is associated with the *mat*− haplotype, and the other is a mutant allele of a gene encoding a cytosolic ribosomal protein.

MATERIALS AND METHODS

Materials. Wild-type *s* strains of both mating type *mat*+ and *mat*− [Fungal Genetics Stock Center (Kansas City, KS) stock nos. 6710 and 6711, respectively] and wild-type *A* (gift of D. Marcou, Institut de Génétique et Microbiologie) were used. The mutation *AS*1–4 was selected from an *s* strain as an informational antisuppressor (20).

Cultures. Cultures, sexual crosses, and life-span measurements were carried out at 27°C as described (8, 21).

mtDNA. Preparation, purification, cloning, restriction analyses, and DNA·DNA hybridization of mtDNA were done by standard methods as described (9, 16, 18).

DNA Sequencing. Nucleotide sequences were determined by the dideoxyribonucleotide chain-termination method using deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate (22) modified to allow direct sequencing of double-stranded DNA (23).

RESULTS

Life-Span and Accuracy of Translation in *P. anserina*. Mutations that increase (*su*, for suppressor) or decrease (*AS*, for antisuppressor) the cytosolic translational accuracy have been described in *Podospora* (see ref. 24 for review). Most of them are point mutations and in several cases it has been shown that they lie in genes encoding proteins of the cytosolic ribosomes (25).

Table 1 gives examples of the effect of such mutations on the life-span of the fungus. Both kinds of mutations (*AS*, which increase, and *su*, which decrease, the translational accuracy) can increase or decrease the life-span. Therefore, the error rate *per se* does not influence the senescence process. The way in which the cytosolic translational apparatus is involved is not clear (21, 26). However, it has been reported that aging in *Drosophila* is associated with a sharp decline in the rate of synthesis of the translational elongation factor 1α (EF1α) (27). Recently, it has been shown that the life-span of the flies is increased when the EF1α gene is overexpressed (28).

Premature Death and Nuclear Genes. We decided to focus our attention on the *AS*1–4 mutant because it has the most

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Table 1. Mean life-span of the wild-type (WT), high (*AS*), and low (*su*) accuracy mutant strains according to their mating type (*mat*+ or *mat*−)

Genotype	Life-span, cm (±SD)				Ribosomal protein
	mat+ strain		*mat*− strain		
WT	37	(4)	21	(3)	—
*AS*1–1	>100		>100		
*AS*1–4	>100		4		S12
*AS*3–1	14	(1.5)	10	(1)	S29
*AS*4–43	54	(4.5)	32	(2.5)	
*AS*6–1	18	(3)	13	(2)	S19
*AS*6–2	>100		>60		S19
*AS*7–2	>100		63	(12)	
*su*1–25	17	(2)	12	(1.5)	
*su*1–31	23	(2)	22	(2)	
*su*1–51	>90		31	(8)	
*su*3–1	>100		>90		S1
*su*3–3	58	(11)	37	(4)	S1
*su*11–1	61	(7)	34	(3)	S8
*su*12–2	94	(11)	/		S7

The life-spans are expressed in centimeters of growth and are the average of 20 measurements from five inoculi issued from four ascospores. The ribosomal proteins indicated were shown to be altered in their electrophoretic mobility in the corresponding mutant strain (see ref. 25). For the *AS*1, *AS*4, and *AS*6 genes, 2, 6, and 3 other alleles have been analyzed, respectively. They all display an increased life-span, whatever the mating type.

clear-cut effect and because it is the only mutant whose effect is dependent on the mating-type haplotype. This particular allele, *AS*1–4, confers a very long life-span to mycelia of *mat*+, while it is responsible for a particularly short life-span in mycelia expressing *mat*− (Table 1). In fact, uninucleate haploid ascospores of the *AS*1–4 *mat*− genotype give rise to mycelia that stop growing 5–6 days after germination under our standard culture conditions. This constitutes the "premature death" phenotype (Fig. 1). Nevertheless, it is possible to use such mycelia for both sexual crosses and DNA purification.

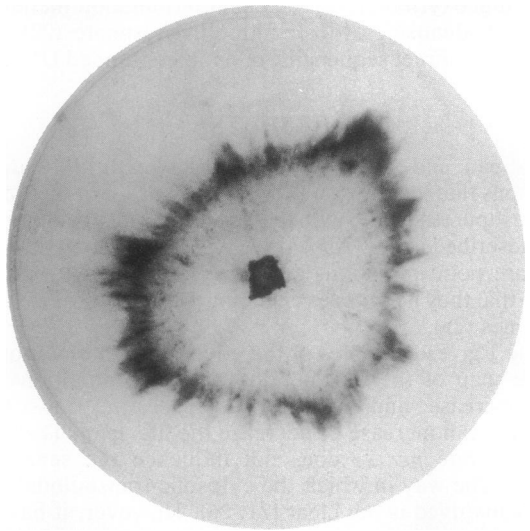

FIG. 1. A mycelium showing the premature death phenotype. A uninucleate ascospore with the *AS*1–4 *mat*− genotype was germinated on a solid germination medium. Twenty-four hours after germination, a piece of this medium containing the germinated ascospore was transferred to the center of a 9-cm-diameter plate filled with solid growth medium. The plate had been incubated 10 days when the picture was taken. The mycelium had stopped growth before reaching the edge of the plate and developed a characteristic dark pigmentation at its border.

The *AS*1–4 *mat*+ mutant has been back-crossed to the wild-type $AS1^+$ *mat*− strain every year from the time of its isolation (20)—i.e., for 14 years. Thus, >500 ascospores carrying the *AS*1–4 mutation and either the *mat*+ or the *mat*− haplotype have been examined. We were unable to detect any recombinant strain displaying either the premature death phenotype while being *mat*+ or a long life-span while being *mat*−. A less extensive study of the $AS1^+$ progenies from the same crosses did not allow us to detect wild-type strains (either *mat*+ or *mat*−) displaying the premature death phenotype. Therefore, we can be sure that the candidate genes (or genes tightly linked to them) are involved in the premature death syndrome.

Structure of mtDNA. As mycelia displaying the premature death phenotype and the senescing mycelia share some common features, particularly a characteristic dark pigmentation observed at the border of the arrested cultures on solid medium (see Fig. 1), we decided to examine mtDNA from strains containing the *AS*1–4 allele. mtDNA from *AS*1–4 *mat*−, *AS*1–4 *mat*+ and the two wild-type $AS1^+$ *mat*+ and $AS1^+$ *mat*− cultures was analyzed with restriction endonucleases and by Southern hybridization. A comparison clearly showed that only strains that contained both the mutant allele *AS*1–4 and the *mat*− haplotype and that exhibited the premature death phenotype contained rearranged mtDNA.

The first conclusions from the analyses illustrated in Fig. 2 can be summarized as follows. mtDNA from *AS*1–4 *mat*+ mycelia contained the wild-type 94-kilobase-pair (kbp) mitochondrial chromosome (restriction map and complete sequence in refs. 29 and 30), while *AS*1–4 *mat*− degenerating mycelia, on the contrary, contained two types of mtDNA molecules in different concentrations, as deduced from the intensities of ethidium bromide-stained electrophoretic bands. One molecule is the wild-type 94-kbp chromosome, which is present at a low concentration, while the other, much more abundant, is a defective chromosome with ≈37 kbp deleted. To this date, mtDNA from more than a dozen prematurely dying mycelia of independent origin—that is, issued from different sexual crosses involving different parental strains—have been purified and analyzed. In all cases, the same restriction pattern was observed. The only variation that could be detected from one culture to another concerns the relative concentration of the two types of molecules.

Determination of the Nucleotide Sequence at the Boundaries of the Deletion. The boundaries of the deletion were determined by restriction analysis to be inside *Eco*RI fragments 4 and 5. A restriction fragment not present in the wild-type strain, and presumed to result from circularization of the deleted chromosome, was visible with most restriction enzymes used (*Eco*RI, *Hae* III, *Eco*RV, *Sac* I, *Bgl* II, *Bam*HI, *Pst* I). We cloned the 5-kbp additional *Bgl* II fragment, which can be easily separated from the other fragments on the gels (Fig. 2). A fine restriction analysis of the cloned fragment confirmed the deletion hypothesis and allowed a precise localization of its boundaries. One, in *Eco*RI fragment 5, is located in a 180-bp segment containing the $tRNA^{Ile}$ and $tRNA^{Ser}$ genes, while the other, in *Eco*RI fragment 4, is located within a few dozen base pairs covering the 5′ junction of intron α, the first intron of the *COI* gene (Fig. 3). This localization was accurate enough to allow direct sequencing of the junction region using double-stranded plasmid DNA as a template and appropriate synthetic oligonucleotides as primers. The recombination event leading to the deletion took place in the 4-bp CAGG sequence, which is repeated 5–9 bp upstream of $tRNA^{Ser}$, in *Eco*RI fragment 5, and at the junction between exon 1 (ending with the first G) and intron α (beginning with the second G) in the gene *COI*, inside *Eco*RI fragment 4 (Fig. 3).

The sequence determination was also carried out on mtDNA extracted from *AS*1–4 *mat*− mycelia, presenting the

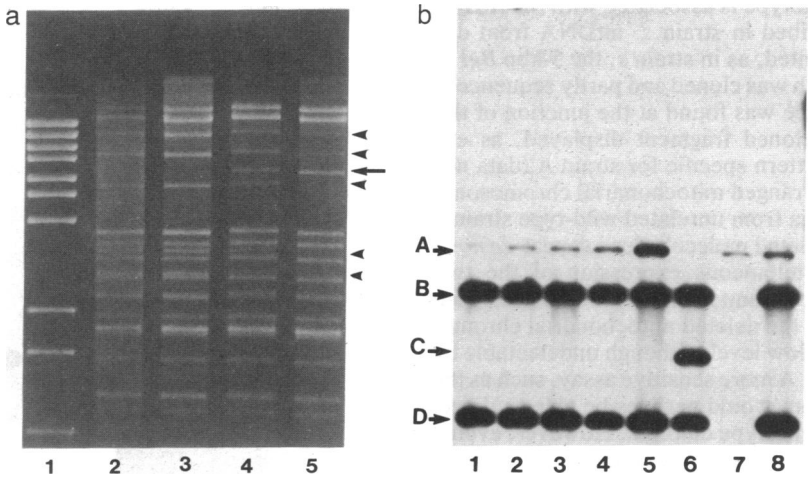

FIG. 2. Analysis of mtDNA from mycelia showing the premature death phenotype. (*a*) DNA digests. Lane 1, molecular weight marker (bacteriophage λ DNA digested by *Bst*EII). Other lanes: *Bgl* II digests of mtDNA from wild-type strain *AS*1$^+$ *mat*+ (lane 2), *AS*1–4 *mat*+ (lane 3), two *AS*1–4 *mt*− strains of independent origin (lanes 4 and 5). Arrow indicates the additional (≈5 kbp) fragment present only in mtDNA from *AS*1–4 *mat*− mycelia. Arrowheads indicate fragments present in the wild-type chromosome that are underrepresented in mtDNA from *AS*1–4 *mat*− mycelia. (*b*) Southern hybridization of mtDNA digested by *Hae* III from strains *AS*1–4 *mat*+ (lanes 1 and 8), *AS*1$^+$ *mat*− (lanes 2 and 3), *AS*1$^+$ *mat*+ (lanes 4 and 5), *AS*1–4 *mat*− (lane 6), and *AS*1$^+$ *mat*− senescent culture (lane 7) (a small amount of this DNA was loaded to show the specific amplification of intron α in this culture). DNA samples from strains of identical genotype were issued from different subclones. The radioactive probe used is a DNA fragment corresponding exactly to intron α. Rows B and D indicate chromosomal *Hae* III fragments containing intron α (1889 and 841 bp for B and D, respectively). Row A indicates the position of free extrachromosomal intron α (2539 bp). Row C is the additional fragment (1270 bp) found only in *AS*1–4 *mat*− cultures (this fragment is entirely included in the 5-kbp *Bgl* II fragment shown in *a*). Autoradiographic films overexposed up to 10-fold failed to reveal a signal at 1270 bp for any DNA other than from *AS*1–4 *mat*− culture.

premature death phenotype, issued from four other ascospores. These ascospores originated from four different crosses in which neither of the parental strains displayed the premature death phenotype (crosses *AS*1–4 *mat*+ × *AS*1$^+$ *mat*−). In the five cases, the deleted molecule presented exactly the same nucleotide sequence at the junction. We have also associated the nuclear genes *mat*− and *AS*1–4 from our *s* reference strain with mitochondria from the unrelated wild-type strain *A* by using the latter as the female parent in two successive crosses. It has been previously shown that mitochondria are not transferred from the paternal parent through sexual crosses in *Podospora* (21, 34). mtDNA from strain *A* differs from that of strain *s* by three additional intervening sequences and several base substitutions leading to a restriction fragment length polymorphism (29, 30, 35). Progenies of these crosses were studied and we observed that

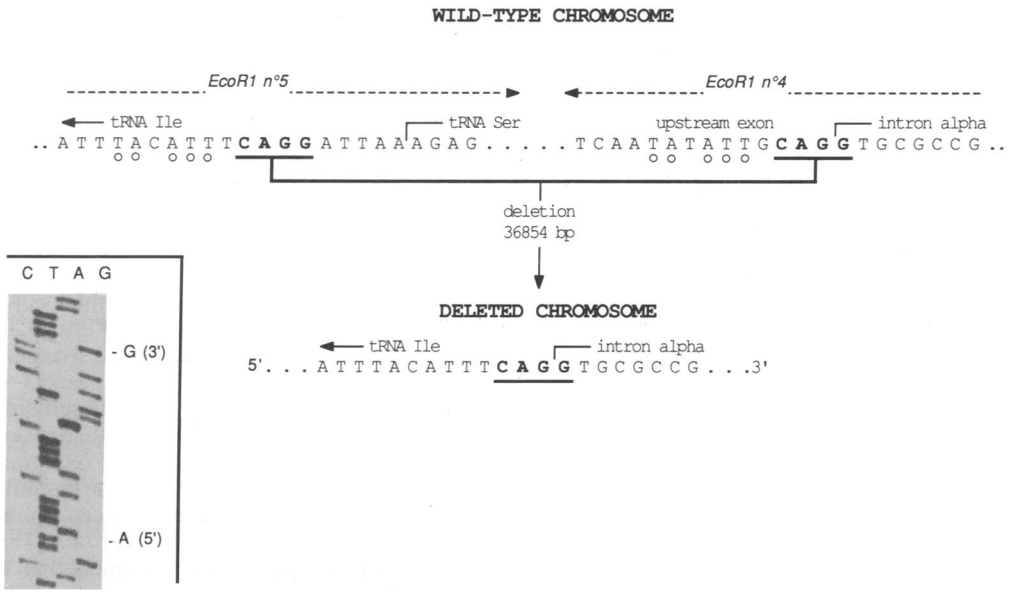

FIG. 3. Structure of the deleted chromosome found in mycelia displaying the premature death phenotype. Mycelia displaying the premature death phenotype contain both the wild-type and a deleted chromosome, which is generally the predominant molecule (see Fig. 2*a*). The nucleotide sequence at the boundaries of the deletion are shown (see refs. 29–31 for complete sequences). The gene encoding tRNA^{Ile} is 159 bp upstream of tRNA^{Ser}. In addition to the underlined repeated sequence CAGG, similarities between sequences are noted by dots under the bases. According to ref. 32, the last 12 bases of the upstream exon constituting IBS1 (from −1 to −6) and IBS2 (from −7 to −12) play an important role in intron splicing by pairing, at the RNA level, with two 6-base intronic sequences EBS1 and EBS2. These sequences are the following: CUGCAA and UGUGUU for EBS1 and EBS2, respectively (33). They would alternatively be able to pair with sequences transcribed from *Eco*RI fragment 5 in the homologous region. (*Inset*) An example of the result of a sequence determination experiment is given. Only the region at the junction of the deleted chromosome is shown. A and G correspond to the 5′ and 3′ nucleotides of the sequence on the bottom line.

the premature death phenotype is associated with the *AS*1–4 *mat*− genotype as described in strain *s*. mtDNA from degenerating mycelia presented, as in strain *s*, the 5-kbp *Bgl* II additional fragment, which was cloned and partly sequenced. Exactly the same sequence was found at the junction of the deleted molecule. The cloned fragment displayed, as expected, the restriction pattern specific for strain *A* (data not shown). As the same rearranged mitochondrial chromosome was found in mitochondria from unrelated wild-type strains, it seems likely that the deleted molecule does appear *de novo* as the result of the simultaneous expression of the two nuclear genes. However, it cannot be excluded that in both wild-type strains *A* and *s* the deleted mitochondrial chromosome is present at a very low level, although undetectable by hybridization (see Fig. 2). A more sensitive assay, such as the polymerase chain reaction, would perhaps be able to detect the deleted molecule in wild-type cultures. However, even if the rearranged molecule was present in the parental strains, the expression of the premature death phenotype requires the presence of both nuclear genes, which would, in that case, either allow the amplification of the preexisting molecule or increase the rate of its formation.

Reversion of the Premature Death Phenotype. We have isolated a spontaneous mutant mycelium that escapes premature death. It was selected as resuming growth from a degenerating stopped mycelium. Its growth rate is regular and low and it is sterile when used as a female parent in sexual crosses. However, studies of different crosses in which it was used as a male parent showed that it contained both of the nuclear genes responsible for the premature death phenotype and that no nuclear mutation acting as suppressor was present. Analysis of the mtDNA from the revertant strain, illustrated in Fig. 4, showed that it is different from the mtDNA of both wild-type and *AS*1–4 *mat*− strains. As judged by restriction analysis and hybridization, the whole of the wild-type genetic information is present but rearranged and organized on two different circular molecules as attested by the presence of two junction fragments. One is the deleted chromosome present in the dying mycelia (see above), and the other contains the remainder of the genome. While the restriction fragments containing both edges of the deletion (the additional 5-kbp *Bgl* II fragment, for example) are present, those containing the wild-type upstream exon–intron α junction are undetectable (Fig. 4). This rearrangement has as a consequence the loss of the cytochrome oxidase function since the gene *CO1* encoding subunit 1 of this enzyme is split by the rearrangement (see Fig. 3). This explains the phenotype of the revertant mycelium in most points similar to that of other mitochondrial mutants of *Podospora*, which were shown to have a part of the *CO1* gene deleted (16, 18). More interestingly, the reversion of the premature death phenotype is correlated with loss of the wild-type upstream exon–intron α junction, which is one of the target sequences for the deletion. This greatly favors the idea that the termination of growth, in premature death, is a direct consequence of this recombination event.

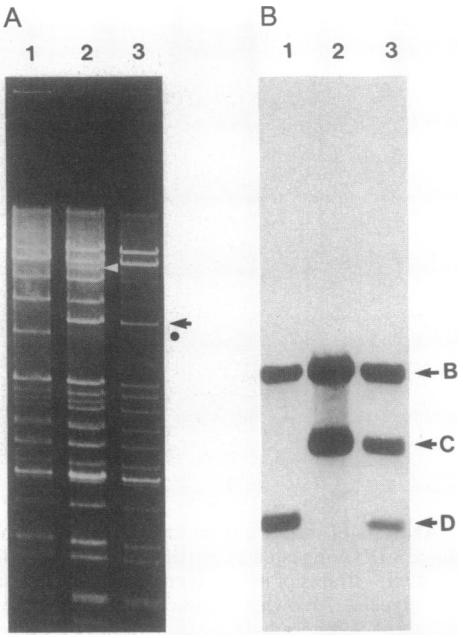

FIG. 4. Comparison of mtDNA from wild-type (lanes 1), revertant (lanes 2), and *AS*1–4 *mat*− cultures (lanes 3) (see also Fig. 2). (*A*) *Bgl* II digests. Note that the mtDNA from the revertant strain (*i*) contains the 5-kbp additional fragment (arrow) joining the edges of the deletion, (*ii*) does not contain the wild-type *Bgl* II fragment 5 (dot) containing the 5′ exon–intron α splice site, and (*iii*) contains another additional fragment (arrowhead) corresponding to the junction fragment of a second circular molecule. (*B*) Southern hybridization of *Hae* III digests (probe, intron α). The following should be noted: (*i*) the presence of the additional fragment C, corresponding to the junction fragment of the deleted chromosome, in both the revertant and the *AS*1–4 *mat*− cultures; (*ii*) the absence, in the revertant mtDNA, of fragment D, which contains the wild-type 5′ exon–intron α splice site.

DISCUSSION

We have discovered that the interaction of two nuclear genes leads systematically to the death of *P. anserina* mycelia at a precise time early in their development. This premature death syndrome is due to an accurate rearrangement of the mtDNA. We have shown that the mtDNA has the same 36,854-bp deletion in all cases studied. One boundary of this deletion is located exactly at the 5′ end of a particular intron that is also involved in the process of senescence in this fungus.

The simplest hypothesis to explain the origin of the defective chromosome is that the deleted molecule results from DNA·DNA recombination involving pairing between imperfectly matched sequences at the sites of the deletion (see Fig. 3). Direct repeats are also found at the sites of deletions in mtDNA in different situations, as petite mutations in yeast (36) or deletions associated with human diseases (3, 4, 37). However, in the case of the premature death syndrome in *Podospora*, the sequence similarity observed between the direct repeats (11-bp sequences differing in two positions; see Fig. 3) is not sufficient to explain the systematic occurrence of the same deletion. Indeed, the 94-kbp mitochondrial genome of *Podospora* contains 65 sequences, 11 bp long, differing from one or the other of the target sequences at two positions or less.

However, one striking feature is the location of one end of the deletion at the 5′ splicing site of intron α. This intron has the unique property of being found as DNA circular molecules particularly abundant in senescent cultures of this fungus (2, 9, 12, 15). It is a class II self-splicing intron and contains an open reading frame able to encode a protein showing similarities with viral and retrotransposon reverse transcriptases (15, 31, 38, 39). Furthermore, homologous introns of yeast have recently been shown to transpose through a reverse transcription step (40). This led us to favor an alternative hypothesis, assuming that the first step in generating the deleted chromosome would be at the RNA level. Other examples of DNA rearrangements in fungal mtDNA have been explained by RNA intermediates such as those mediated by the Varkud plasmid in *Neurospora* (41, 42). Loss of introns at the DNA level was supposed to result from recombination involving a reverse-transcribed mature RNA (16, 43–45). In *Podospora*, examination of the sequence immediately upstream of the 5′ end of the deletion showed that it contains potential intron binding sites (32)

(IBS1 and IBS2), which would be able to pair with intronic EBS1 and EBS2 complementary sequences in intron α (see Fig. 3) and to play an acceptor function in a transesterification reaction, the reverse of the first step of splicing (46). Recently, Mörl and Schmelzer (47) showed that a class II intron is able to mediate, at the RNA level, site-specific recombination events giving rise, among the products, to a structure identical to that we observed at the DNA level at the junction of the deleted chromosome. This recombinant RNA molecule results from an "exon exchange," at the 5′ end of the intron, of the normal upstream exon with an alternative RNA molecule containing an IBS1 motif.

Genetic studies strongly suggest that the candidate genes (*AS*1–4 and *mat*−), or less probably genes tightly linked to them, are responsible for the mtDNA rearrangement. The two mating-type haplotypes have been cloned in one of our laboratories (M.P.), and >100 kbp encompassing the *mat*+ and *mat*− are now available. Isolation of the *AS*1 gene has also been undertaken through genomic library screening with oligonucleotides deduced from partial sequences of the S12 ribosomal protein.

It remains to be understood how the products of the nuclear genes are so efficiently active in promoting such a precise recombination event. One possibility would be that these genes are involved in mtRNA metabolism and would favor the transligation event we have assumed to give rise to the recombined RNA molecule.

The involvement of a ribosomal protein defect in the premature death phenotype could be discussed in the context of the control of mitochondrial intron splicing by the translational apparatus. Such a control has been exemplified by the effect of mutations in aminoacyl-tRNA synthetase genes on mitochondrial RNA splicing both in *Neurospora* (48) and in yeast (49). More recently, it was discovered that in yeast the nuclear *SUV*3–1 mutation is involved in both posttranscriptional modification and translation of mtRNAs (50).

Finally, we would like to stress the striking similarities, at different levels, between the premature death phenotype in *Podospora* and a familial mitochondrial myopathy, which is under stringent control by a nuclear gene recently described in humans (6). This disease, transmitted as an autosomal dominant trait, is associated with the presence of deleted mtDNA molecules in heteroplasmic muscular cells. Whatever the mechanisms involved in premature death, a better knowledge of nuclear-encoded proteins that control the mitochondrial genome stability might yield insight into human diseases in which mtDNA rearrangements are involved.

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