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Mutations in Genes Encoding the Mitochondrial Outer Membrane Proteins Tom70 and Mdm10 of *Podospora anserina* Modify the Spectrum of Mitochondrial DNA Rearrangements Associated with Cellular Death

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Tom70 and Mdm10 are mitochondrial outer membrane proteins. Tom70 is implicated in the import of proteins from the cytosol into the mitochondria in *Saccharomyces cerevisiae* and *Neurospora crassa*. Mdm10 is involved in the morphology and distribution of mitochondria in *S. cerevisiae*. Here we report on the characterization of the genes encoding these proteins in the filamentous fungus *Podospora anserina*. The two genes were previously genetically identified through a systematic search for nuclear suppressors of a degenerative process displayed by the *AS1-4* mutant. The PaTom70 protein shows 80% identity with its *N. crassa* homolog. The PaMdm10 protein displays 35.9% identity with its *S. cerevisiae* homolog, and cytological analyses show that the *PaMDM10-1* mutant exhibits giant mitochondria, as does the *S. cerevisiae mdm10-1* mutant. Mutations in *PaTOM70* and *PaMDM10* result in the accumulation of specific deleted mitochondrial genomes during the senescence process of the fungus. The phenotypic properties of the single- and double-mutant strains suggest a functional relationship between the Tom70 and Mdm10 proteins. These data emphasize the role of the mitochondrial outer membrane in the stability of the mitochondrial genome in an obligate aerobe, probably through the import process.

The maintenance of intact mitochondrial genomes in eukaryotic organisms is controlled by a complex (genetic and environmental) system. A number of nuclear genes involved in this process have been identified in the yeast *Saccharomyces cerevisiae*, because this facultative aerobe is suitable for the selection of mutations that alter mitochondrial DNA (mtDNA) content. In contrast, none of the genes that control the stability of the mtDNA and/or the relative transmission of wild-type and rearranged mtDNA molecules have yet been characterized in higher eukaryotes, which are obligate aerobes.

Filamentous fungi may bridge the gap between these two situations. Like multicellular organisms, they are obligate aerobes and can display heteroplasmic states in which intact and rearranged mitochondrial genomes coexist. Like yeasts, they are suitable systems for genetic selection and molecular analyses. The filamentous fungus *Podospora anserina* has additional interesting properties. First, the growth of the wild-type strains is limited by a systematic degenerative process called senescence (28), which is associated with the amplification of circular molecules (senDNAs) of particular regions of the mitochondrial chromosome (for reviews, see references 12 and 14). Second, each wild-type isolate exhibits a characteristic life span (22). Consequently, genes whose products are directly or indirectly involved in the generation or transmission of mtDNA alterations can be identified by mutations which modify either the life span and/or the spectrum of the senescence-specific mtDNA rearrangements. *AS1* is one of these genes. Indeed, the *AS1-4* mutation leads to the accumulation of specific deleted mtDNA molecules during cellular death and modifies life span (2, 6, 29). The latter effect is influenced by a second gene (of unknown function), which is tightly linked to the mating-type (*mat*) locus and displays two natural alleles. The *AS1-4 mat*− strains die very early (premature death), while the *AS1-4 mat*+ strains live longer (6). Since the *AS1* gene encodes a protein of the cytosolic ribosomes, the effect of the *AS1-4* mutation on the mtDNA is indirect (11). To identify genes more directly involved in the accumulation of the Δ molecules, suppressors allowing *AS1-4 mat*− strains to escape the premature death syndrome were screened. Strains bearing only the suppressor mutations were further recovered through genetic crosses (7).

In this study, we characterize two of these suppressor genes (*RGS43* and *RGS27*). Sequence comparisons provide evidence that *RGS43* encodes the Tom70 mitochondrial outer membrane protein involved in the import of proteins from the cytosol into the mitochondria (for reviews, see references 8, 21, and 25). For *RGS27*, both sequence comparison and the presence of giant mitochondria in the mutant strain strongly suggest that the gene is the homolog of the *S. cerevisiae MDM10* gene, which also encodes a component of the mitochondrial outer membrane (35). Mutations in the two genes lead to modified life spans and to new mitochondrial genome structures at the time of cellular death. The phenotypic properties of the mutant and double-mutant strains suggest that the Tom70 and Mdm10 proteins are functionally related.

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TABLE 1. Phenotypic properties of *PaTOM70-1*, *PaMDM10-1*, and *PaTOM70-1 PaMDM10-1* (*mat*+ and *mat*−) mutant strains

Genotype	Phenotypic property[d]	Growth rate (% of the wild-type)[a] under the indicated condition			
		27°C	35°C	18°C	+CAP (1 g/liter)
PaTOM70-1					
mat+	Very spindly	83	NM[c]	100	63
mat−	Very spindly	83	50	100	63
PaMDM10-1					
mat+	Tiny	83	54	100	83
mat−	Tiny	83	70	100	83
PaTOM70-1 PaMDM10-1					
mat+	Tiny	65	0	0	ND[b]
mat−	Tiny	65	0	0	ND

[a] Growth rates of the wild-type strains (per day) were as follows: 6 mm (*mat*+ and *mat*−) at 27°C, 6.5 mm (*mat*+) and 7.5 mm (*mat*−) at 35°C, 1.6 mm (*mat*+ and *mat*−) at 18°C, and 5.5 mm (*mat*+ and *mat*−) in the presence of 1 g of CAP per liter.

[b] ND, not determined.

[c] NM, not measurable (because growth stopped very early).

[d] Germination. All strains had few aerial hyphae.

MATERIALS AND METHODS

Media, strains, and life span measurements. Standard growth conditions and media were previously described (13). Growth and life span measurements were performed on minimal synthetic medium (M2). Sensitivity towards chloramphenicol (CAP) was measured on cornmeal extract medium supplemented with CAP (1 g/liter). All strains derived from the wild-type *S* strain. The library was constructed from the *s* strain, which is isogenic with the *S* strain except for two incompatibility genes (5). The *mat* locus has two natural alleles, *mat*+ and *mat*−. The *AS1-4* mutant was isolated as an antisuppressor (translational high-fidelity mutant) (26). Its effect on life span and mtDNA structure in *mat*+ and *mat*− backgrounds was reported previously (2, 6). The *rgs43* and *rgs27* mutants were selected by Contamine and Picard (7) as suppressors able to delay the premature death phenotype displayed by *AS1-4 mat*− strains.

The *rgs43 rgs27* (*mat*+ and *mat*−) double-mutant strains were obtained by crossing an *rgs43 mat*− strain with an *rgs27 mat*+ strain. Tetrad analysis permitted the isolation of putative double-mutant strains that were further checked by crosses with wild-type strains and recovery in the progeny of the *rgs43* and *rgs27* mutants.

The life span of a strain is defined as the mean length of growth of parallel cultures between the point of inoculation and the arrested edge of the dead culture (22). Life span was measured as previously described (7).

Nucleic acid analysis. DNA analysis and PCR experiments were performed by standard methods (30). The mtDNA of young and senescent mycelia was extracted by a miniprep procedure (20). The nuclear DNA was extracted according to the method of Cummings et al. (9).

Cosmids and plasmids. The genomic library (a kind gift of C. Barreau and V. Berteaux-Lecellier) used for the transformation experiments was constructed from an *s mat*+ strain in the cosmid vector pMOcosX. This integrative vector carries as a dominant selective marker the bacterial hygromycin B resistance gene under the control of the *cpc1* promoter of *Neurospora crassa* (24). Subcloning of the *RGS43* and *RGS27* genes was performed with the pBlueScript SK(+) vector (Stratagene).

Cloning and sequencing procedures. The *RGS43* and *RGS27* wild-type genes have been cloned by complementation of the mycelial phenotype (Table 1) displayed by the *rgs43* and *rgs27* mutants by the SIB selection method (1). The library contained about 6,000 cosmids from the entire genome, which were divided into 60 pools. Transformation experiments were performed as previously described (4).

The fourth pool tested with the *rgs43* recipient strain produced 202 hygromycin B-resistant transformants, 2 of which presented a wild-type mycelial phenotype on the protoplast regeneration medium and also on M2 medium. When the *rgs27* mutant strain was used as a recipient, hygromycin B-resistant transformants displaying a wild-type phenotype (2 of 92) were obtained with the 26th pool tested. Two successive rounds of SIB selection allowed the isolation of the cosmids carrying the wild-type alleles of *RGS43* and *RGS27*. One transformant obtained in each experiment was crossed with the corresponding mutant strain. Cosegregation of resistance to hygromycin B and wild-type phenotypes was verified in the progeny. To localize the genes within the cloned DNA, the cosmids were completely digested with one of several restriction enzymes (38). Each restriction mixture associated with the pMOcosX reporter vector was used to transform the mutant strains. This method can be used to identify which enzymes inactivate and which enzymes do not inactivate the gene.

A 3.7-kb *Bam*HI-*Sal*I fragment complementing the *rgs43* mutant was isolated from the original cosmid. Digestion of this fragment with *Sst*I gave two fragments of 2.4 and 1.3 kb, but neither complemented the mutant strain. Restriction site *Sst*I was therefore assumed to be located inside the gene. The *rgs43* mutant allele was recovered in a 5-kb *Sal*I-*Sal*I fragment (containing the 3.7-kb *Bam*HI-*Sal*I fragment) after a genomic DNA minibank of the mutant strain was screened. For *RGS43* wild-type and mutant alleles, a total of 2,156 bp lying on both sides of the *Sst*I site were sequenced on both strands by the method of Sanger et al. (31), with a set of oligonucleotides as primers.

A 7.5-kb *Bam*HI-*Sma*I fragment complementing the *rgs27* mutant was subcloned from the original cosmid. Digestion of this fragment by *Sal*I produced two fragments of 4.3 and 3.2 kb, neither of which displayed a complementing capacity. A total of 2,143 bp extending on both sides of the *Sal*I site present in the cloned 7.5-kb *Bam*HI-*Sma*I fragment were sequenced on both strands. The sequence of the mutant allele of the gene was determined after PCR amplification of various fragments overlapping the gene. The PCR fragments were directly sequenced according to the method of Khorana et al. (19). The presence of the mutation was ascertained by sequencing two independent PCR fragments on both strands.

Cytology. Wild-type, mutant, and double-mutant strains were grown on solid M2 medium at 27°C for 2 days. Then the cultures were examined immediately or shifted to 35°C for 40 h before being examined. Staining was performed with the mitochondrion-specific dye 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide (DASPMI) purchased from Sigma, according to the procedure described by McConnell et al. (23). Filaments taken from the cultures were mixed with a drop of a solution of 25 μg of DASPMI per ml on microscope slides and observed immediately with a fluorescence microscope.

Nucleotide sequence accession numbers. The nucleotide sequences of *PaTOM70* and *PaMDM10* have been submitted to the EMBL data library under accession no. Y14750 and Y14749, respectively.

RESULTS

The *RGS43* gene encodes the mitochondrial outer membrane translocase Tom70. The *RGS43* and *RGS27* wild-type genes were cloned by complementation of the phenotype entailed by their mutant alleles (see Materials and Methods).

Comparison of the amino acid sequence deduced from the nucleotide sequence of the *RGS43* wild-type gene with those in the EMBL data bank revealed 80% identity with the protein Mom72/NcTom70 of *N. crassa* (25, 37) and 36% identity with Mas70/ScTom70 of *S. cerevisiae* (15, 25) (Fig. 1). The coding sequence of *RGS43* is interrupted by two introns of 61 and 116 bp whose positions are indicated in Fig. 1. Tom70 is a mitochondrial outer membrane protein that functions as a receptor for protein import into the mitochondria (for reviews, see references 8 and 21). The *RGS43* gene is probably the *P. anserina* homolog of *TOM70*. Therefore, the gene and the encoded protein have been renamed *PaTOM70* and PaTom70, respectively.

The mutation *PaTOM70-1*, responsible for suppressing the premature death syndrome exhibited by the *AS1-4 mat*− strain, was identified after the sequencing of the affected region from the genomic DNA of the mutant strain (see Materials and Methods). This frameshift mutation alters the last 97 amino acid residues of the protein (Fig. 1). In the yeast and *N. crassa* proteins, the corresponding residues are part of a large hydrophilic domain exposed to the cytosol (16, 32).

***RGS27* is probably the homolog of the *S. cerevisiae MDM10* gene.** The nucleotide sequence of the *RGS27* wild-type gene showed two open reading frames present in two different phases. A BLAST sequence homology search of protein databases revealed that the putative polypeptides encoded by these open reading frames displayed significant similarity to *S. cerevisiae* Mdm10. Mdm10 is a mitochondrial outer membrane protein required for normal mitochondrial morphology (35). We relied on these data to identify a 62-bp canonical intron in the *RGS27* gene (see Fig. 2 for its position). Figure 2 shows an optimized amino acid alignment of Mdm10 and the Rgs27

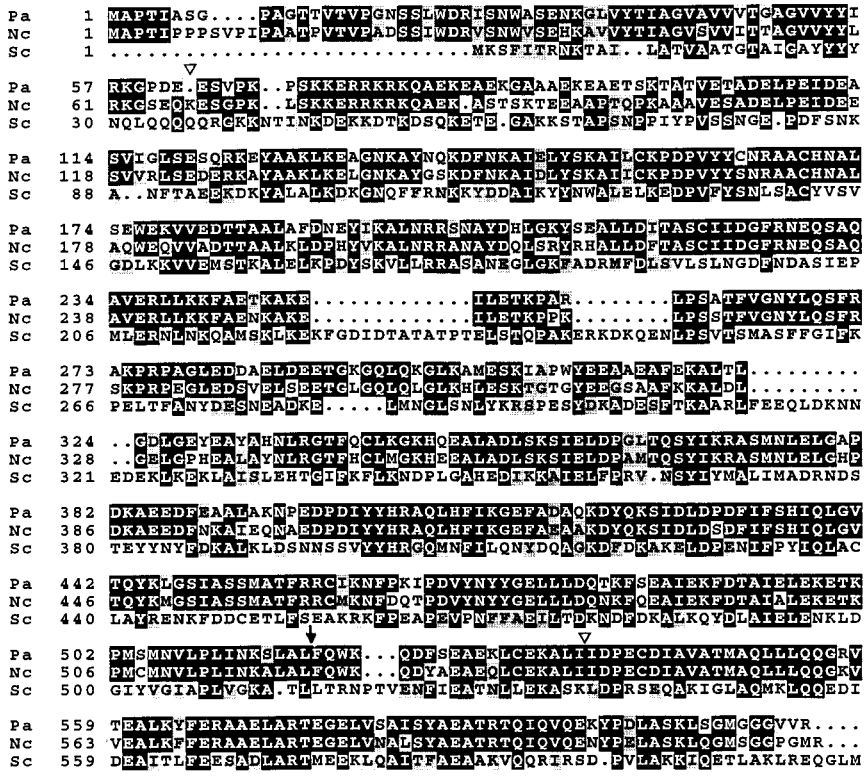

FIG. 1. Comparison of the predicted amino acid sequence of *P. anserina* Rgs43 (Pa; 614 amino acids [aa]) with those of *N. crassa* Tom70 (Nc; 619 aa) (37) and *S. cerevisiae* Tom70 (Sc; 617 aa) (15). The alignment was obtained by the PILEUP algorithm. Gaps (indicated by dots) were introduced to maximize alignment. Identical amino acids are in black boxes, and similar amino acids are in gray boxes. The positions of the two introns in the *P. anserina RGS43* gene are indicated by triangles, and the position of the (−1) frameshift mutation in the *RGS43* gene is indicated by an arrow.

protein in which they have 35.9% amino acid identity. This percentage is within the average range of those commonly found between homologous proteins fulfilling the same function in filamentous fungi and yeasts. This suggests that Rgs27 is a homolog of Mdm10, a conclusion strengthened by cytological observations which revealed the presence of giant mitochondria in the *rgs27* mutant strain (see below). The *RGS27* gene and its encoded protein were thus renamed *PaMDM10* and PaMdm10, respectively.

The sequence of the mutant gene (*PaMDM10-1*) was estab-

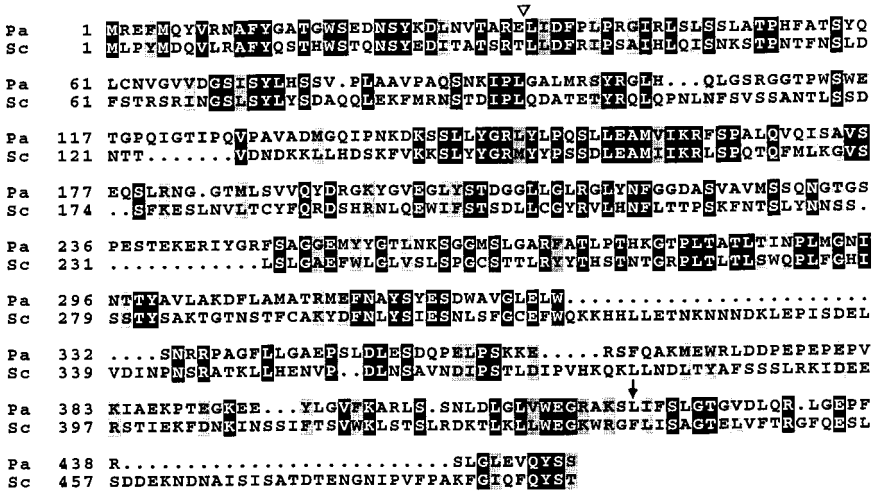

FIG. 2. Comparison of the predicted amino acid sequence of *P. anserina* Rgs27 (Pa; 448 aa) with that of *S. cerevisiae* Mdm10 (Sc; 494 aa) (35). The alignment was obtained by the PILEUP algorithm. Gaps (indicated by dots) were introduced to maximize alignment. Identical amino acids are in black boxes, and similar amino acids are in gray boxes. The position of the intron in the *P. anserina RGS27* gene is indicated by a triangle, and the position of the missense mutation in the *RGS27* gene is indicated by an arrow.

TABLE 2. Life spans[a] of wild-type and mutant strains

Genotype	Life span (length of growth [cm]) ± standard deviation		
	Wild-type	*PaTOM70-1*[a]	*PaMDM10-1*
mat−	9.1 ± 0.6	260 ± 143	4.7 ± 2
mat+	8.4 ± 0.5	121 ± 106	11.1 ± 5.4

[a] Data for *PaTOM70-1* are rough estimates since only 1 of 20 *mat*+ and 3 of 20 *mat*− cultures were still alive.

lished after PCR amplification of the mutant DNA with oligonucleotide primers flanking the gene. The mutation responsible for the suppression of premature death is a missense that changes a leucine into a proline. This alteration occurs 28 amino acids upstream of the C terminus of the protein (Fig. 2).

***PaTOM70-1* and *PaMDM10-1* have common pleiotropic effects.** The two mutations display several phenotypic properties, listed in Table 1. First, both mutations lead to an alteration in germinating mycelium (germination phenotype). Second, they cause a decrease of the mycelium aerial hyphae, especially at the beginning of growth. Third, they increase sensitivity towards CAP (1 g/liter) on cornmeal extract medium. Fourth, the two mutants exhibit a thermosensitive phenotype at 35°C, whereas their growth compared to that of the wild-type strain is impaired only slightly at 27°C and not at all at 18°C. Similarly, the yeast mutants disrupted for either *TOM70* or *MDM10* grow slowly on a nonfermentable carbon source at temperatures between 23 and 30°C and not at all at 37°C (27, 35, 37). Fifth, both *P. anserina* mutations lead to a modified life span compared to that of the wild-type strains (Table 2). *PaTOM70-1* strongly increases the life span, whatever the *mat* background. *PaMDM10-1* clearly decreases the life span in the *mat*− context.

The five phenotypic properties of the *PaTOM70-1* and *PaMDM10-1* strains were complemented through transformation with cosmids carrying the wild-type copy of the relevant gene.

The number of giant mitochondria seen in the *PaMDM10-1* single mutant is strikingly increased in the *PaMDM10-1 PaTOM70-1* double mutant. The mycelium of *P. anserina* consists of branched filaments subdivided by septa pierced by a central pore which allows the passage of nuclei, vacuoles, and mitochondria toward the growing tips and to the new branches formed during its development. Examination of mitochondria stained with the mitochondrion-specific dye DASPMI in wild-type, *PaTOM70-1*, *PaMDM10-1*, and *AS1-4* mutant strains showed that the number of mitochondria is highly variable along all growing mycelia, ranging from 1 to 5 in thin branches and older parts to over 50 in the larger young compartments and the tips. Wild-type mitochondria undergo frequent branching or fragmentation, but their snakelike morphology (Fig. 3A) and mean sizes remain unchanged at temperatures from 27 to 35°C; however, at 18°C, mitochondria are clearly smaller.

When the *PaMDM10-1* mutant is grown at 35°C, all hyphal compartments contain giant mitochondria which are either spherical, oval, or tadpole shaped (Fig. 3B). When the *PaMDM10-1* mutant is grown at 27°C, the distribution of these large mitochondria is more heterogeneous: in most compartments, they are mingled with "normal" mitochondria, but some compartments contain only "normal" mitochondria. No giant mitochondria are seen in cultures grown at 18°C. The number and size of these giant mitochondria increase significantly in cultures grown at temperatures from 27 to 35°C and decrease when cultures are returned to a permissive temperature. Giant

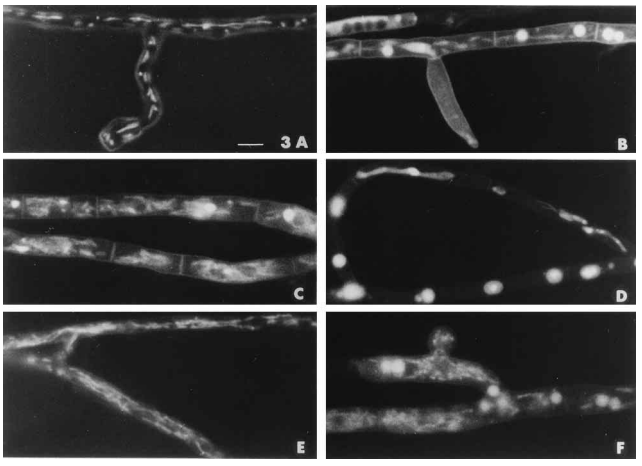

FIG. 3. Phenotypes of mitochondria stained with DASPMI as seen in wild-type and mutant strains grown at 35°C. (A) Wild-type snakelike mitochondria of a mycelial branch. (B) Three compartments of *PaMDM10-1*: two contain giant mitochondria, and the third, which corresponds to a young branch, is empty. (C) *PaTOM70-1*, showing a large number of snakelike mitochondria clustered in the central portion of the compartments mixed with enlarged mitochondria. (D) The *PaTOM70-1 PaMDM10-1* double mutant shows only giant mitochondria which are either round or snakelike. The number of mitochondria per compartment is lower than those in both single mutants shown in panels B and C. (E) *AS1-4* mitochondria are thinner and more numerous than those in a wild-type strain. (F) *PaMDM10-1 AS1-4* double mutant shows round giant mitochondria mixed with numerous mitochondria of the *AS1-4* phenotype. Bar, 5 μm.

mitochondria are able to pass through the septal pores, but their movement seems impaired because, as seen in Fig. 3B, young branches often remain empty. Interestingly, at high temperatures, *PaTOM70-1* also shows giant mitochondria (Fig. 3C). Although fewer and smaller than the abnormal mitochondria of *PaMDM10-1*, these mitochondria are clearly larger than the structures seen in the corresponding wild-type culture. Furthermore, the "normal" mitochondria of *PaTOM70-1* appear to be clustered in the center of most compartments, an arrangement that was never observed in wild-type cultures (compare Fig. 3C and A).

For additional controls, we screened the *AS1-4* mutant (from which both *PaTOM70-1* and *PaMDM10-1* mutants were selected) and a wild-type senescent culture at 35°C. None showed enlarged mitochondria. On the contrary, both displayed only small mitochondria, which accumulated in large numbers in all compartments of the mycelium as illustrated in Fig. 3E for *AS1-4*. However, their shapes differed: *AS1-4* mitochondria were thin and snakelike, while mitochondria in the wild-type senescent mycelia were round (data not shown). All compartments of the *PaMDM10-1 AS1-4* double mutant (Fig. 3F) contained a mixture of giant mitochondria similar in size to those seen in the single *PaMDM10-1* mutant and numerous thin mitochondria characteristic of the *AS1-4* single mutant (compare Fig. 3F and E). In contrast, the *PaTOM70-1 AS1-4* double mutant clearly exhibited the single *PaTOM70-1* mutant phenotype, namely a mixture of enlarged and small mitochondria that were not as thin as the *AS1-4* mitochondria (data not shown). Finally, mitochondria from *PaMDM10-1* and *PaTOM70-1* transgenic strains carrying the relevant wild-type gene grown at 35°C showed only wild-type phenotypes (data not shown).

When the *PaTOM70-1 PaMDM10-1* double mutants were shifted to a temperature of 35°C, all compartments of their mycelia contained almost solely giant snakelike or spherical mitochondria (Fig. 3D). The development of these giant mi-

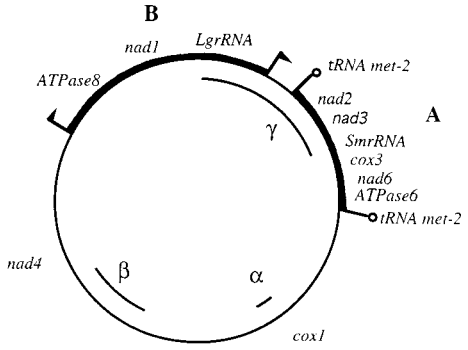

FIG. 4. Positions of the different mtDNA regions amplified in wild-type and *PaMDM10-1* senescent cultures. α, β, and γ correspond to the regions amplified in senescent wild-type cultures. Regions A and B (thick lines) correspond to the regions amplified in senescent *PaMDM10-1* cultures. The genes encompassed by these regions are indicated (10). Region A is bounded by two copies of the *tRNAmet-2* gene (indicated by ♀); region B is bounded by two 30-bp direct repeats (indicated by ▶).

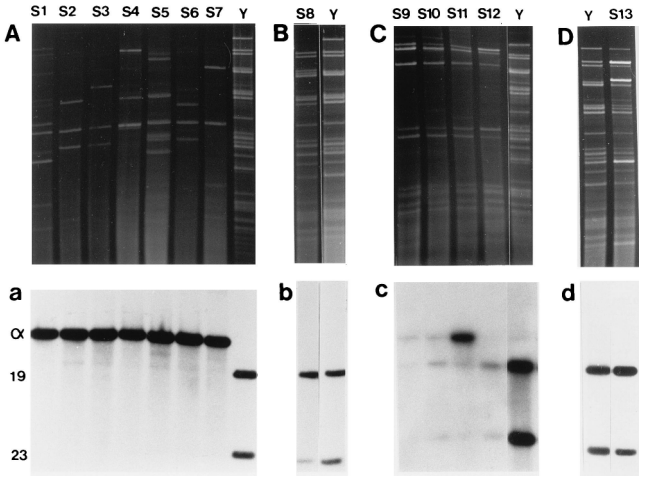

FIG. 5. *Hae*III restriction patterns of mtDNA extracted from young (Y) or senescent (S) cultures (uppercase letters) and identification of senDNAα by hybridization with a specific probe (lowercase letters). (A and a) One young and seven independent senescent cultures of wild-type strains. S1, S2, and S6 are *mat*+; S3, S4, S5, and S7 are *mat*−. (B and b) One young (Y) and one senescent (S8) culture of the *PaTOM70-1 mat*− strain. (C and c) One young and four independent senescent cultures from *PaMDM10-1* strains. S9 and S10 are *mat*−; S11 and S12 are *mat*+. (D and d) One young and one senescent culture (S13) of the *PaTOM70-1 PaMDM10-1 mat*− strain. The cloned intron α was used as a probe in the hybridization experiments. Intron α contains one *Hae*III site. Probe α reveals the *Hae*III restriction fragments 19 (1.8 kb) and 23 (1.2 kb) and, when present, a 2.5-kb fragment corresponding to senDNAα (indicated by α).

tochondria could have occurred by the rearrangement of preexisting smaller structures; there are always fewer per compartment than the corresponding wild-type mitochondria, and a rapid return to smaller units was observed when the double-mutant strain was shifted back to a permissive temperature.

***PaTOM70-1* and *PaMDM10-1* modify the spectrum of the mtDNA rearrangements associated with the senescence process.** The senescence syndrome (28), which occurs in all wild-type strains of *P. anserina*, is associated with major rearrangements of mtDNA, in particular with the amplification of some regions as head-to-tail, circular, double-stranded molecules—the senDNAs. The senDNAs arise from three distinct regions (α, β, and γ) of the mitochondrial chromosome (for a review, see reference 12) (Fig. 4). SenDNAα is systematically recovered in large amounts from senescent cultures of wild-type strains (Fig. 5A and a), and its monomer corresponds precisely to the first intron (intron α) of the *cox1* gene. SenDNAβ and SenDNAγ are recovered frequently but not systematically from senescent cultures. Unlike senDNAα, these two species are formed with monomers of variable sizes and termini. Each species, however, has a common core. Examples of mtDNA restriction patterns characteristic of wild-type senescent cultures are shown in Fig. 5A.

An analysis of the mtDNA of seven *PaTOM70-1* senescent cultures revealed two interesting features. First, in all these senescent cultures, senDNAα was either undetectable by hybridization (Fig. 5b) or present in low amounts. Second, surprisingly, in two cases out of seven, the restriction pattern showed high amounts of defective molecules complementary to the particular senDNAβ present at low amounts in the same culture (Fig. 5B). In the hundreds of senescent cultures of the wild-type strain that have been studied in our laboratory (about 70% contained senDNAβ), molecules complementary to senDNAβ have never been found amplified. However, their presence had been detected previously by PCR in cultures that contained senDNAβ (18). Thus, the *PaTOM70-1* mutation allows the accumulation of a particular class of defective molecules which are undetectable or present in very low amounts in wild-type strains.

An even more noteworthy result was obtained with *PaMDM10-1*. Twenty-two independent senescent cultures were examined. Substantial amounts of senDNAα were detected in only a few cases (Fig. 5C and c). Moreover, 18 of the 22 cultures displayed exactly the same amplified fragments (Fig. 5C and c). Of the fragments amplified in the four other cultures (not shown), some were similar to the previous ones, but others were not. Because the complete sequence of the mitochondrial chromosome of *P. anserina* is known (10), we precisely identified the common fragments and established that as circular molecules, they corresponded to the amplification of two distinct regions of the mtDNA, designated A and B (Fig. 4). PCR experiments performed with appropriate divergent oligonucleotides as primers and subsequent sequencing of the PCR products established that the circularization point of each class of amplified molecules was exactly the same in mtDNA preparations extracted from six independent cultures (data not shown). The breakpoints of each of the two defective genomes A and B are bounded precisely by direct repeats, one copy of which is found in the defective molecule. In case A, these repeats correspond to the 71-bp *tRNAmet-2* gene which is duplicated in the mitochondrial genome of *P. anserina*; in case B, they correspond to a 30-bp noncoding sequence. The presence of A molecules in one senescent culture of the wild-type strain was previously reported (10). The identification by PCR in *PaMDM10-1* cultures of molecules deleted precisely for sequence A or B (not shown) indicates that defective molecules A and B are generated by intramolecular crossovers occurring between the direct repeats. Such a mechanism was previously shown to be involved in the generation of the senDNAβs (18). In conclusion, the *PaMDM10-1* mutation is responsible for the reproducible accumulation of two specific defective mitochondrial genomes of 14.8 and 21.2 kb (Fig. 4) which have been sporadically observed (A molecules) or not yet detected (B molecules) in senescent wild-type strains. Interestingly, using PCR followed by direct sequencing of the products, we have shown that A and B molecules are almost always present in small amounts in young as well as in senescent cultures of wild-type strains. This fact suggests that

the *PaMDM10-1* mutation is involved in the accumulation of the defective A and B molecules rather than in their genesis.

Properties of the *PaTOM70-1 PaMDM10-1* double-mutant strains. The phenotypic properties of the double-mutant strains are listed in Table 1. These strains display the same germination phenotype as the *PaMDM10-1* single mutant and exhibit few aerial hyphae, as do the two single mutants. Their growth rate is reduced compared to that of each single mutant; they grow more slowly at 27°C and not at all at 35°C. It is noteworthy that, unlike the single mutants, which grow as well as the wild-type at 18°C, the double-mutant strains are sensitive to cold and do not grow at all at this temperature. As reported above, the double-mutant mycelia contain almost exclusively giant mitochondria at high temperatures (Fig. 3).

Preliminary data concerning the double-mutant life span obtained from six *mat*− and three *mat*+ isolates (one subculture from each isolate) suggest very heterogeneous behavior. The three *mat*+ isolates died in the range of the single mutant *PaMDM10-1 mat*+ isolates (life span, 6.5 to 16 cm). Among the six *mat*− isolates, three displayed life spans in the range of the *PaMDM10-1 mat*− mutant (4 to 7 cm), while the other three grew to 40 cm without displaying any indication of death. Such heterogeneous values were also observed for the single-mutant strains (Table 2).

The mtDNAs of eight independent senescent cultures of the *PaTOM70-1 PaMDM10-1* (*mat*− and *mat*+) double mutants were analyzed. The five *mat*− cultures displayed nearly identical restriction patterns that revealed the presence of two major populations of chromosomes: one probably corresponded to the wild-type genomes, while the other, found in larger amounts, corresponded to the defective A genome previously observed in the *PaMDM10-1* mutant (Fig. 4D). The restriction patterns of the three *mat*+ cultures also reflected a mixture of intact and overrepresented defective genomes. In one case (not shown), the latter appeared equivalent to the defective B genome, also characteristic of *PaMDM10-1*; the defective genomes were not identified in the two other cases. The hybridization presented in Fig. 4d is representative of what is observed for all cultures analyzed and indicates that senDNAα is nearly undetectable in *PaTOM70-1 PaMDM10-1* senescent cultures.

DISCUSSION

An unexpected screening procedure for *TOM70* and *MDM10* in *P. anserina*. Two mutations identifying two different genes (*RGS43* and *RGS27*) in *P. anserina* were identified previously as allowing the *AS1-4 mat*− mutant strain to escape premature death syndrome (7). This degenerative process is linked to the accumulation of specific mitochondrial deleted genomes (Δ molecules) (2).

We have cloned and sequenced these two genes and shown that the Rgs43 protein is highly homologous to Tom70 of *N. crassa* and that the Rgs27 protein has significant similarity to Mdm10 of *S. cerevisiae*. *TOM70* was identified in *N. crassa* and *S. cerevisiae* because of the previous characterization of the mitochondrial outer membrane protein it encodes (15, 27, 37). This protein is a component of the receptor complex involved in the import of proteins from the cytosol to the mitochondria (36; for reviews, see references 8 and 21). Mutants were subsequently obtained in *S. cerevisiae* by disruption of the *TOM70* gene (27, 37). *MDM10* was cloned in *S. cerevisiae* by complementation of a mutant strain (*mdm10-1*) displaying aberrant mitochondrial distribution and morphology at 37°C. This gene also encodes a mitochondrial outer membrane protein whose function is not yet clear but which is suspected to interact with the cytoskeleton (35).

We have also shown that the *rgs27* mutant, like the *mdm10* mutants of *S. cerevisiae* (35), displays giant spherical mitochondria along with normal-sized organelles at both 27 and 35°C. Strikingly, the *rgs43* mutant also exhibits (but only at 35°C) enlarged mitochondria, a feature not yet reported for the *TOM70* mutants. Such giant mitochondria are not found in wild-type strains of *P. anserina* at any growth temperature. Finally, we have observed that the *rgs43* and *rgs27* mutants are heat sensitive, as are the *tom70* and *mdm10* yeast mutants.

Altogether, the sequence homologies and the striking effects of the mutations on mitochondrial morphology provide indirect but convincing evidence that the Rgs43 and Rgs27 proteins are the *P. anserina* homologs of Tom70 and Mdm10 and that their functions are similar to those reported in *N. crassa* (Tom70) and yeast (Tom70 and Mdm10).

The striking phenotypic similarities of the *PaTOM70-1* and *PaMDM10-1* mutants provide new insights into the Mdm10 function. The *PaMDM10-1* mutant was obtained in the experiment that provided the *PaTOM70-1* mutant; it was thus logical to submit the two mutants to the same phenotypic analysis. This led to the discovery of interesting features in both mutants, which, to our knowledge, have not been reported in yeast. Besides the giant mitochondria shown by the *PaTOM70-1* mutant at 35°C (see above), we would like to stress three main points. First, both mutants display an increased sensitivity towards CAP, an inhibitor of the mitochondrial protein synthesis. Second, in addition to their effects in the *AS1-4* background, the two mutations modify the life span of the wild-type (*AS1*) strains. Finally, cellular death of both mutants is associated with the presence of specific rearranged mitochondrial genomes, different from those commonly observed in wild-type and *AS1-4* strains. In either mutant, cellular death is not correlated with the massive amplification of senDNAα that is characteristic of senescent wild-type strains.

These data prompted us to examine the *PaTOM70-1 PaMDM10-1* double-mutant strains. As expected, some of their phenotypic properties are similar to those displayed by the single mutants. More interestingly, the double-mutant strains exhibit increased heat sensitivity associated with a spectacular increase in giant mitochondria at 35°C. Finally, it is noteworthy that the double-mutant strains display an absolute cold-sensitive phenotype that is not exhibited by the single mutants. As reported for other biological systems (17), the similar properties of the *PaTOM70-1* and *PaMDM10-1* mutants, along with the particular features of the double mutants (especially the synthetic lethality at 18°C), suggest a functional relationship between the Tom70 and Mdm10 proteins.

The giant mitochondrial phenotype displayed by the *PaTOM70-1* mutant at 35°C suggests that a defect in Tom70 may alter mitochondrial shape (at least at high temperatures), probably because of the deficient import of Mdm10 or other mitochondrial proteins. Thus, the role of Tom70 in the mitochondrial morphology would be indirect. The striking phenotypic features shared by the *PaTOM70-1* and *PaMDM10-1* mutants suggest that protein import could also be altered in the *PaMDM10-1* mutant. This may be caused directly or indirectly by the mutation. One possibility is that the Mdm10 protein is directly involved in the import process. The second possibility is that the protein per se is not involved but that the abnormal morphology of the mutant mitochondria indirectly causes a defect in this process. These assumptions must be critically tested in *S. cerevisiae* and *N. crassa*, which are presently the most suitable models for the study of the relationships between mitochondrial shape and protein import. In any case, an al-

TABLE 3. Working model of the effects of impaired translational and/or import apparatus on the mitochondrial genome

Cytosolic apparatus or mtDNA	Protein class(es) in nuclear background of[a]:			
	AS1 PaTOM70	*AS1-4 PaTOM70*	*AS1-4 PaTOM70-1*	*AS1 PaTOM70-1*
Protein synthesis	A,B,C	A,b,c	A,b,c	A,B,C
Protein import	A,B,C	A,b,c	a,b,c	a,B,c
mtDNA[b]	senDNAα	Δ molecules	None	Other variants

[a] A, B, and C are three classes of nucleus-encoded proteins devoted to mitochondria. Uppercase letters denote normal expression, and lowercase letters denote a defect in synthesis and/or import (see text for details). Δ molecules, deleted mtDNA molecules.

[b] Corresponding to defective molecules accumulated at time of death.

tered import is the simplest explanation of the heat sensitivity of the two mutants (if optimal import is required at high temperatures) along with their sensitivity towards CAP (if mitochondrial ribosome assembly is impaired in import mutants).

Mitochondrial outer membrane proteins, cytosolic translation, and the stability of the mitochondrial genome. It is quite obvious that the fate of the mitochondrial genome (in terms of replication, recombination, and transmission, etc.) depends on proper equilibrium between numerous proteins which have to be synthesized in the cytosol and imported into the organelle. We can therefore predict that a defect in the cytosolic translational apparatus and/or import apparatus will lead to an instability of the mtDNA. This prediction is exemplified in *P. anserina*. The *AS1* gene encodes a cytosolic ribosomal protein which does not enter the mitochondria (11). The accumulation of the defective mitochondrial Δ molecules in *AS1-4* strains is thus clearly an indirect effect of the *AS1-4* mutation. These defective molecules were shown to be cryptic, i.e., constitutively present at a low level, in young wild-type cultures (33). The properties of mutants altered in different ribosomal proteins (including *AS1-4*) led to the proposal that specific alterations of ribosomes can cause disequilibrium in the synthesis of nucleus-encoded proteins addressed to mitochondria, leading to modified suppressivity of cryptic defective molecules in premature death and in the senescence process as well (34). The same rationale can be applied to a defect in the import process due to the *PaTOM70-1* (*PaMDM10-1*?) mutation, which probably modifies the relative amounts of nucleus-encoded proteins which have to reach the mitochondrial compartment. Such an effect could antagonize the *AS1-4* (translational effect) and lead to the accumulation of noncanonical defective mtDNA molecules in an *AS1* context. Notably, it was recently reported that the yeast *mdm10* mutant cells readily produced respiration-deficient (rho$^-$ or rho^o) cells at rates much higher than that of the wild-type parental strain (3).

In our working model, outlined in Table 3, we consider three classes of proteins, A, B, and C, which are synthesized in the cytosol and imported into the mitochondria. Proteins B and C are supposed to be underexpressed in the *AS1-4* background due to a particular defect of the cytosolic ribosomes. Proteins A and C are assumed to require the Tom70 protein for efficient import into the mitochondria. Thus, according to the relevant nuclear backgrounds, different amounts of these proteins are eventually found inside the mitochondria, leading to the altered fate of the mtDNA and its variants. This defect in the import process also explains the increased life span of the *PaTOM70-1* mutant, probably through a slow rate of genesis and/or accumulation of the relevant mtDNA variants. A future challenge will be to identify the genes which encode the relevant classes of proteins and thus directly control mtDNA stability.

Beyond the fungi. The identification of human genes that affect the structural integrity of the mitochondrial genome is a popular topic. Genes encoding factors involved in the replication and transcription of mtDNA are considered to be the most interesting candidates (39). Our results show that genes which encode proteins of the mitochondrial outer membrane can play a striking role in the stability of the mtDNA, at least in some cases by impairing the import process. Such genes must therefore be considered valuable candidates in the study of human disorders associated with the rearrangement of mtDNA.

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