Two Copies of \textit{mthmg1}, Encoding a Novel Mitochondrial HMG-Like Protein, Delay Accumulation of Mitochondrial DNA Deletions in \textit{Podospora anserina}

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In the filamentous fungus \textit{Podospora anserina}, two degenerative processes which result in growth arrest are associated with mitochondrial genome (mitochondrial DNA [mtDNA]) instability. Senescence is correlated with mtDNA rearrangements and amplification of specific regions (senDNAs). Premature death syndrome is characterized by the accumulation of specific mtDNA deletions. This accumulation is due to indirect effects of the \textit{AS1-4} mutation, which alters a cytosolic ribosomal protein gene. The \textit{mthmg1} gene has been identified as a double-copy suppressor of premature death. It greatly delays premature death and the accumulation of deletions when it is present in two copies in an \textit{AS1-4} context. The duplication of \textit{mthmg1} has no significant effect on the wild-type life span or on senDNA patterns. In an \textit{AS1}° context, deletion of the \textit{mthmg1} gene alters germination, growth, and fertility and reduces the life span. The \textit{3mthmg1} senescent strains display a particular senDNA pattern. This deletion is lethal in an \textit{AS1-4} context. According to its physical properties (very basic protein with putative mitochondrial targeting sequence and HMG-type DNA-binding domains) and the cellular localization of an \textit{mthmg1}-green fluorescent protein fusion, \textit{mthmg1} appears to be a mitochondrial protein possibly associated with mtDNA. It is noteworthy that it is the first example of a protein combining the two DNA-binding domains, AT-hook motif and HMG-I boxes. It may be involved in the stability and/or transmission of the mitochondrial genome. To date, no structural homologues have been found in other organisms. However, \textit{mthmg1} displays functional similarities with the \textit{Saccharomyces cerevisiae} mitochondrial HMG-box protein Abf2.

The maintenance of the mitochondrial genome is essential for most eukaryotic organisms. In humans, in addition to sporadic cases of deletion, a growing number of familial diseases are associated with mitochondrial DNA (mtDNA) deletions (see reference 29 for a review). In sporadic diseases, the most frequent deletion (common deletion) involves about one-third of the mtDNA molecule. The deletion molecules can be detected at very low levels in healthy individuals (18) and in oocytes (11). In patients, cells are usually heteroplasmic (with a variable amount of intact mtDNA) (see reference 29 for a review), but the mechanism leading to accumulation of defective molecules in these diseases remains unknown. To date, four genes implicated in familial diseases have been cloned and characterized. They encode a thymidine phosphorylase (34), an adenine nucleotide translocator (26), the mtDNA polymerase γ (49), and a novel mitochondrial protein, Twinkle, suspected to be a helicase (46). Although all these proteins are probably involved in mtDNA metabolism, the reasons for which mutations in the relevant genes cause the accumulation of multiple deletions of mtDNA remain poorly understood.

Lower eukaryotes, in which classical and molecular genetics are simpler, have been very useful as model systems to study diverse biological problems, particularly mtDNA stability and transmission, as for example the budding yeast \textit{Saccharomyces cerevisiae} (see reference 16 for a review). The filamentous fungus \textit{Podospora anserina} also appears to be a good model system. In this obligate aerobe, two degenerative processes associated with mtDNA instabilities have been characterized: senescence (40) and premature death (6). Senescence is a spontaneous process associated with mtDNA rearrangements and amplifications of specific regions (senDNAs) (see reference 7 for a review). Premature death is linked to the accumulation of mtDNA molecules which have lost a specific part of the genome (6, 41). Some similarities can be found with human diseases characterized by the accumulation of the so-called common deletion (29). In \textit{P. anserina}, these deletions can be detected in trace amounts in young wild-type cultures (41), but they accumulate only in the presence of the nuclear mutation \textit{AS1-4} or \textit{AS1-5}. These mutations affect a gene encoding a cytosolic ribosomal protein (20). Consequently, their effect is likely indirect via cytosolic translation.

To identify genes more directly involved in the accumulation of mtDNA deletions and to better understand the phenomenon, two classical strategies have been used. First, after mutagenesis, suppressors allowing \textit{AS1-4} strains to escape premature death syndrome were screened. Suppressors were obtained that either delay or abolish the accumulation of the deletion (14). It was shown previously that two recessive suppressors were localized in genes encoding proteins of the mitochondrial outer membrane (25). Several dominant mutations in at least four loci were also identified previously (14). However, the corresponding genes are not yet cloned. The second strategy was a search for multicopy suppressors. No autono-
mously replicative multicopy plasmids are available in *P. anserina*. Transformation usually leads to random ectopic single-copy integration of the transforming DNA containing a cloned gene. Only a twofold increase in copy number may thus be expected. Nevertheless, it appeared to be sufficient to promote a suppressor effect, at least in one case (30). We thus began systematic transformation experiments on an AS1-4 strain with a cosmid library from *P. anserina* genomic DNA and sought transformants exhibiting an increased life span. In this paper, we describe the first gene identified with this method, *mthmg1*, which, in two copies, delays premature death. Our results strongly suggest that mtHMG1 is an mtDNA-binding protein belonging to the HMG family which may indeed be involved in mitochondrial genome maintenance, stability, or transcription.

**MATERIALS AND METHODS**

*P. anserina* strains and media. *P. anserina* is a heterothallic ascomycete whose life cycle and general methods for genetic analysis have been described elsewhere (23). The AS1 mutations were first described as informational antisuppressors (38). The *rmp1* gene is tightly linked to the mat locus and has two natural forms, *rmp1*-1, linked to *mat*−, and *rmp1*-2, linked to *mat* + (previously called *rmp−* and *rmp+*, respectively) (13). This gene has been shown to play a key role in AS1-4 and AS1-5 longevity. The *rmp1*-2 allele (13) greatly delays the cessation of fungal growth compared to the *rmp1*-1 allele (dominant allele) (life span, 80 versus 2 cm), but in both cases the mtDNA deletions still accumulate at the time of death (13). In this study, we mainly used *rmp1*-1 strains in which premature death could be rapidly observed. Mutations increasing the life span of the AS1-4 *rmp1*-1 strain have been described previously (14). *rgs12* is one of these mutations: the life span of the AS1-4 *rmp1*-1 *rgs12* strain is over 90 cm, and the accumulation of the mtDNA deletion characteristic of premature death is not observed at the point of death.

*rgs12* mutations altering translation and characterized as informational anti-suppressors were used in this study: AS1-1 and AS1-2, probably altering modification enzymes of a ribosomal component; AS4-3, lying in the gene for the eEF-1A elongation factor; AS6-2, AS6-5, and AS12-2PR8, localized in ribosomal protein genes (see reference 17 for a review; 45).

All media (cormical extract [MR], minimal synthetic [M2], and germination [G]) were as described by Esser (23). For transformation experiments, M1 (23) or RG (26 plus sucrose) was used.

**Transformation of *P. anserina***. Transformation experiments were performed as previously described (20). Transformants were selected on medium (M1 or RG) containing hygromycin B (from Roche Diagnostics or Life Technologies) at 100 mg/liter or phleomycin (from Sigma) at 10 mg/liter, depending on the selective marker used.

**Life span measurements.** Life spans are usually measured on M2 medium in 25- or 35-cm glass tubes with twice the usual amount of agar, for at least 10 independent strains or several subcultures from four to six strains with a given genotype. The cultures are grown at 27°C in the dark. The life span of a strain is defined as the mean length of growth of parallel cultures between the point of inoculation from a germinated spore and the arrested edge of the dead culture. Growth arrest is easily detected due to the pigmentation changes of dead mycelium. The growth arrest is defined as the mean length of growth of parallel cultures between the point of inoculation from a germinated spore and the arrested edge of the dead culture. Growth arrest is easily detected due to the pigmentation changes of dead mycelium. To check for the presence of a premature death suppressor in progenies of crosses, AS1-4 or AS1-5 spores were tested on petri dishes.

**Library construction.** The genomic library used for transformation experiments was constructed from an *rgs12 rmp1*-1 strain (14). High-molecular-weight genomic DNA was digested with the *SalI* restriction enzyme. The fragments were ligated in the cosmids vector pMCOsoX, whose dominant selective marker is the bacterial hygromycin B resistance gene (*hph*) under the control of the *Neuros- poncrassa* cssp1 promoter (35). The library was divided into 50 pools of 96 cosmid-containing bacteria and stored in microwell plates.

**Vectors and bacterial strains.** Cloning and subcloning of DNA fragments from *P. anserina* were performed in the *PUC18*, *pBluescript* SK (+) or KS (+) (Stratagene), or pBC-Hygro (43) vector. *E. coli* DH5α (24) was used for genomic library construction, and *CM10α* (10) was the recipient strain for all recombinant plasmids.

**Isolation of genomic DNA and total RNA.** Genomic DNA for PCR or hybridization experiments was extracted as described previously (20). mtDNA from senescent strains was extracted by the rapid method (28) and analyzed by *HaeIII* digestion and Southern blotting. Implants were removed at approximately 1 cm from the margin of dead mycelia. The specific probes used to reveal *senDNA* and *senRNA* have been described previously (5). Total RNA was extracted by the method of Lecellier and Silber (28) for 5’ rapid amplification of cDNA ends (RACE) or by the guanidinium thiocyanate-phenol-chloroform method (20) for cDNA preparation and Northern blotting.

**mRNA analyses.** To verify the presence of the intron, cDNA was constructed by reverse transcription-PCR as previously described (20) with primers 3 and 6 (Fig. 1A) and sequenced. The 5’ end of *mthmg1* mRNA was determined from approximately 3 μg of total RNA (a kind gift of Hervé Lalanne) by two different methods (5’-RACE) or by the guanidinium thiocyanate-phenol-chloroform method (20) for cDNA preparation and Northern blotting.

**Sequencing the *mthmg1* gene region.** The Smal fragment was completely sequenced by using the ABI PRISMS Ready Reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) with a 573A automatic sequencer (Applied Biosystems). Several subclones from pBSSmal-4.5 were generated by using various restriction enzymes and sequenced on both ends with universal and reverse primers. Synthetic primers were then deduced to fill the gaps and complete the sequence on both strands (Fig. 1A). The corresponding wild-type region of genomic DNA was amplified by PCR (Taq DNA polymerase from Applied Oncor) with several pairs of primers (1 and 2, 3 and 4, 3 and 10, and 9 and 8 [Fig. 1A]) and sequenced after purification, by using border or internal primers.

**mthmg1 inactivation.** A null allele of *mthmg1* (Δ*mthmg1*) was constructed by transcriptional inactivation of an 866-bp *PshI*-BglII fragment (Fig. 1A) with a *PvuI*-BamHI fragment containing the phleomycin resistance gene (*ble*) from the *pBPaBle* plasmid (16). This plasmid was digested with *NruI* and *BspHI*, and the deletion-containing fragment was used (Fig. 1A). To circumvent the possibility of lethality of the *mthmg1* deletion, we transformed an AS1-4 *rmp1*-1 strain bearing an ectopic *mthmg1* copy previously obtained by transformation with the *pBSRv2.2* plasmid. Putative deletion transformants were selected as exhibiting a premature death phenotype. This was indeed the case for 20 of 308 phleomycin-resistant transformants analyzed. We analyzed their molecular structure by PCR and Southern blot hybridization. Two primer pairs were tested (two in the *mthmg1* gene and two in the *rmp1* gene) with several pairs of primers (1 and 2, 3 and 4, 3 and 10, and 9 and 8 [Fig. 1A]) and sequenced after purification, by using border or internal primers.
as the beginning of the GFP gene, and a 3′ GFP primer after the vector NorI site (5′-CGCATCTCCTCCTCCAACAGCAACAGTCCTCCTCCACTCGG-3′). In parallel, an mtHMG1 DNA fragment was amplified by using primers 7 and 4 (Fig. 1A). After purification, 1 μl of each PCR fragment was mixed and subjected to a second round of PCR with primers 3 and 5′ GFP primer to amplify a fusion fragment by overlap extension. The resulting fragment was digested with BclI and NorI and exchanged with the corresponding fragment in the pBSBBg plasmid, yielding a heterokaryotic plasmid. Hygromycin-resistance and a short life span and since these phenotypes are recessive, we used a AS1-5 rmp1-1 allele cloned in pBC-Hygro by using the BglII and PstI fragment from pmtHMG1-GFP containing the end of the ORF fused with GFP coding sequence, and the III PCR fragment digested with BglII and PstI. After sequencing, the final construction was subcloned in pB-Hygro by using the NorI and SalI vector sites, yielding pBC-mtHMG1-GFP. This plasmid was introduced by transformation into AS1 or AS1-5 rmp1-1 protoplasts. Since the ΔmtHMG1 strain displays altered growth and viability under stress and since these phenotypes are recessive, we used a heterokaryotic recessive ΔmtHMG1 leu1+ mat–/– mutation and an mtHMG1 leu1+ mat+ strain. This heterokaryotic strain shows a wild-type growth and life span, thus allowing preparation of protoplasts. The AS1-5 rmp1-1 strain, while manifesting the premature death phenotype, exhibits a slightly increased life span than does the ASI-4 rmp1-1 strain, thus permitting the preparation of homokaryotic protoplasts. Hygromycin-resistant transformants complemented for the growth defect of ΔmtHMG1 or selected for an increased life span of ASI-5 rmp1-1 were purified by crossing them with the wild-type strain and were submitted to cytological observation.

mtHMG1 overexpression. A gene fusion with the gpd (glyceraldehyde-3-phosphate dehydrogenase) promoter of P. anserina (39) was generated as follows. A gpd promoter-containing fragment was amplified from pGIHA (2) with PU and HOP2 primers, by using Pfu DNA polymerase. A gpd-ΔmtHMG1 fusion primer (5′-CTCTCCTCCAACAGCAACAGTCCTCCTCCACTCGG-3′) was designed to amplify the ΔmtHMG1 ORF in conjunction with primer 2. One microliter of each PCR fragment was used in a second-round PCR with primers PU and 2, and the resultant purified fragment was digested with HindIII and inserted in pBReH in the HindIII site. In the recombinant colonies, the correct orientation of the gpd fragment was confirmed by PCR directly on the colonies with primer 8 and PU and by plasmid extraction and digestion with EcoRI. The gpd-ΔmtHMG1 plasmid was sequenced before use to ensure that no mutagenesis was introduced during the construction.

Miscellaneous procedures. Most purifications of DNA fragments from either PCR mixtures or agarose gels were performed with GFX PCR DNA and the GelBand Purification kit (Amersham Pharmacia Biotech). Standard procedures for Southern and Northern blotting and hybridizations on nylon membranes (Amersham or Appligene) were used. Low-stringency hybridization was performed at 50°C with a final wash in 2× SSC (1× SSC is 0.15 M NaCl plus 0.0015 M sodium citrate) at 55°C. Probes were prepared with a random primer kit (T7 Quick Prime; Amersham). For Northern hybridizations, the intensity of each band was recorded by using the ImageQuant software (Molecular Dynamics).

Cytology. Wild-type and mutant strains were grown on solid M2 medium for 3 days at 27°C. Cultured filaments were mixed with a drop of water or M2 liquid medium on microscope slides and observed by fluorescence microscopy. Staining with the mitochondrial-specific dye DASPMI (2-[4(dimethylaminostyryl)-1-methylpyridinium iodide; Sigma) was performed as previously described (25) at a final concentration of 25 μg/ml. Strains expressing mtHMG1-GFP fusion protein were observed without fixation after incubation with DAPI (4′,6′-diamidino-2-phenylindole; 1 μg/ml; Boehringer) to visualize the mtDNA and nuclei. Observations were performed on a Zeiss Axioskop photomicroscope. Fluorescence images were captured by a charge-coupled device Princeton camera system and processed with Photoshop 5.0 LE and Microsoft Photo Editor.

Nucleotide sequence accession number. The nucleotide and protein sequences have been submitted to EMBL-EBI (accession no. AJ316007).

RESULTS

The mtHMG1 gene delays premature death when present in two copies. The SIB selection method (1) was used to identify a cosmid carrying a premature death suppressor by transformation of an ASI-4 rmp1-1 recipient strain with a P. anserina genomic DNA library (see Materials and Methods). One transformant among 125 analyzed from one pool exhibited the expected phenotype (increased life span). Two successive rounds of SIB selection isolated a cosmid (8F5) able to greatly increase the life span of an ASI-4 rmp1-1 strain (Table 1) (more than 100 cm rather than 2 cm). The relevant cosmid was subcloned according to the method of Turcq et al. (47) using pB-Hygro as a cotransformation plasmid. A 2.2-kb EcoRV fragment (Fig. 1A) was finally shown to be sufficient to confer premature death suppression. We demonstrated (data not shown) that the cloned gene contained in the EcoRV DNA fragment did not correspond to the rgs12 mutant allele from which the library was made but that it was able to suppress premature death when present in two copies in an ASI-4 rmp1-1 strain. It was called mtHMG1 because of its sequence characteristics (see below). Addition of an ectopic copy of this gene does not modify the ASI-4-associated phenotypes, except for life span.

mtDNAs from dying cultures of different purified transformants obtained with the 8F5 cosmid in an ASI-4 rmp1-1 context were analyzed. As shown in Fig. 2, they show a pattern similar to that of a dying ASI-4 rmp1-1 strain. The mtDNA deletions are either of the classical DΔ (6) or the previously described DΔ-type, with no amplification of specific senDNAs. Thus, two copies of mtHMG1 much delay, but do not

<table>
<thead>
<tr>
<th>Strain and transformant</th>
<th>Life span (cm)</th>
<th>mtDNA pattern</th>
</tr>
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<tbody>
<tr>
<td>ASI+ mtHMG1+</td>
<td>14.8 ± 1</td>
<td>senDNAs</td>
</tr>
<tr>
<td>ASI+ mtHMG1+</td>
<td>2 ± 0.4</td>
<td>mtDNA deletions</td>
</tr>
<tr>
<td>B</td>
<td>14.6 ± 2.8</td>
<td>senDNAs</td>
</tr>
<tr>
<td>F</td>
<td>11.6 ± 3.7</td>
<td>senDNAs</td>
</tr>
<tr>
<td>A</td>
<td>&gt;110</td>
<td>mtDNA deletions</td>
</tr>
<tr>
<td>B</td>
<td>&gt;110</td>
<td>mtDNA deletions</td>
</tr>
<tr>
<td>C</td>
<td>&gt;90</td>
<td>mtDNA deletions</td>
</tr>
<tr>
<td>ASI+ ΔmtHMG1</td>
<td>3.8 ± 1</td>
<td>senDNAγ</td>
</tr>
<tr>
<td>ASI–4 mtHMG1 (mtHMG1+)</td>
<td>~2.3</td>
<td>Not tested</td>
</tr>
<tr>
<td>ASI–4 mtHMG1 (mtHMG1+)</td>
<td>12.9 ± 3.7</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

* All the experiments were performed in the rmp1-1 context in which premature death could be rapidly observed. The strains with two copies of mtHMG1 (additional ectopic copy indicated in parentheses) were obtained from several independent transformants containing either the 8F5 cosmid (A, B, and C), or the pBS5ma5.5 plasmid (F), both of which fully complemented the ΔmtHMG1 null allele (ΔmtHMG1).

* Life spans were measured as described in Materials and Methods for at least 10 cultures.

* In the ASI–4 context, for A and B, only one culture died in each case at 32.5 and 51.5 cm, respectively. For C, five cultures died at 23.5, 45, 65, 90, and 97 cm, respectively. All other cultures were still growing when the measurements were stopped. Results of analysis of mtDNA of some of these dying cultures are shown in Fig. 2.

* Data were obtained with the ectopic copy of transformant F.

* Mean of only three cultures.
abolish, the accumulation of the mtDNA deletions in an AS1-4 rmp1-1 context.

Low-stringency hybridization showed that mthmg1 is unique in a wild-type strain (data not shown). The addition of a second copy of the mthmg1 gene has no striking effect in an AS1/H11001 context: the life span of an mthmg1/H11001 (mthmg1/H11001) strain (Table 1), as well as the mtDNA patterns of dying strains, is similar to that of a wild-type strain with accumulation of senDNAs (Fig. 2).

mthmg1 encodes a putative mtDNA-binding protein. The 2.2-kb Eco RV fragment contains an ORF with one putative intron (Fig. 1A and 3). The presence of this intron was established by reverse transcription-PCR and sequencing. The size of the mRNA was estimated by Northern blotting as approximately 1.7 kb. A major transcription start was localized by 5’ RACE methods (see Materials and Methods) 65 bases upstream of the ATG codon, in a pyrimidine-rich region, as is usual for transcription starts in fungal genes (3). According to the ORF and mRNA lengths, the 3’ untranslated tail seems rather long (estimated at 700 nucleotides). However, a copy truncated at the Eco RV site, located 450 bp after the stop codon, is still able to promote premature death suppression (Table 1).

The deduced 316-amino acid (aa) protein (Fig. 3) is very basic (pI 11.15) and displays a putative N-terminal mitochondrial targeting sequence according to the work of Claros and

![Diagram of mthmg1 chromosomal region](image-url)
Vincens (12). Overall, the N-terminal region is very rich in hydrophobic and basic residues, is completely lacking in acidic residues, and can fold into an amphiphilic \(\alpha\)-helix structure (aa 15 to 32, Fig. 4). MitoProt II analysis (http://www.mips.biochem.mpg.de/proj/medgen/mitop/) predicts a cleavage site at position 72. Thus, mtHMG1 is probably a mitochondrial protein. mtHMG1 contains two very hydrophilic regions, the first just after the predicted cleavage site between aa 70 and 120 and the second in the C-terminal region (Fig. 4). Interestingly, according to the SMART program (42) (http://www.smart.embl-heidelberg.de/), mtHMG1 exhibits putative domains found in high-mobility-group proteins (Fig. 3 and 4) (see reference 9 for a review and references therein). The sequences encompassing aa 147 to 211 and 244 to 310 can be rather well aligned with the consensus for HMG-1 boxes (4). An AT-hook motif (aa 111 to 123) containing the central typical GRP tripeptide surrounded by basic residues is present, displaying a well-conserved pattern. The mtHMG1 protein thus displays characteristics of two subfamilies of HMG DNA-binding proteins.

Database searches indicate that mtHMG1 has no clear structural homologue known other than a recently identified ORF of unknown function in the \(N.\ crassa\) sequencing project (MIPS, on cosmid 9a26, ORF ncu02695.1, and WICGR, contig 3.138) (30% identity; data not shown).

\(\text{AS1}^+\) strains in which mtHMG1 is either absent or overexpressed display an altered phenotype. In order to examine the role of \(\text{mtHMG1}\), the resident gene was deleted as described in Materials and Methods. The \(\text{PshAI-BclI}\) fragment was replaced with the phleomycin resistance gene (\(\text{ble}\)) as a selection marker (Fig. 1A). To eliminate the possibility of lethality of the null allele, we transformed an \(\text{AS1-4 mmp1-1}\) strain bearing an
ectopic copy of \textit{mthmg1} (long life) and screened for phleomycin-resistant transformants which have recovered a premature death phenotype (life span of about 2 cm). Eight transformants were finally recovered, displaying the expected phenotype and DNA structure. Three were crossed with a wild-type strain to eliminate the ectopic copy and ascertain the phenotype associated with the inactivation of \textit{mthmg1}. In an \textit{AS1}/H11001 background, the \textit{mthmg1} mutation shows a very altered phenotype.

FIG. 3. Nucleic acid and deduced protein sequences of the \textit{mthmg1} gene. The sequence of the EcoRV fragment is shown. For the nucleic acid sequence, relevant restriction sites used (see Materials and Methods) are shown above the sequence. Oligonucleotide primers used to determine the 5' end of the messenger and to check the intron position are indicated with dashed arrows above the sequence. Vertical arrows indicate the major (\s) and minor (\s) transcription starts. For the deduced protein sequence, the DNA-binding motifs (AT-hook and HMG-1 boxes) are underlined with the characteristic, usually highly conserved amino acids in boldface (see reference 2). The complete sequence of the \\textit{SmaI} 4.5-kb fragment has been submitted to EMBL (accession no. AJ316007).
ROLE OF TWO COPIES OF mthmg1 IN Podospora anserina

**FIG. 4.** Characteristics of the mtHMG1 protein. mtHMG1 protein was analyzed with the DNA Strider 1.3 (31) and SMART (42) software programs. (A) Acid-basic map. (B) Hydrophobic pattern according to the work of Kyte and Doolittle. (C) Helical wheel with 95° angles depicting a predicted amphipathic $\alpha$ helix between aa 15 and 32. Basic residues (R) are in boldface italic, and hydrophobic residues are boxed. The dashed line indicates the hydrophilic and hydrophobic portions of the helix. (D) Functional domains. MTS, putative mitochondrial targeting sequence; AT, AT-hook domain; HMG, regions similar to HMG boxes. The amino acid positions are given above the diagram. The position of the amphipathic $\alpha$ helix is shown in dark gray.

**mthmg1** is essential in the **ASI-4** context. According to segregation analysis of the progeny of the crosses, **ASI-4 mthmg1** spores are formed but do not germinate. Sometimes, only one or two very short filaments emerge from the spore, but growth arrests very soon, even if the spore is transferred on growth medium and under a variety of germination temperatures. **mthmg1** is thus lethal in association with the **ASI-4** mutation. Introduction of an **mthmg1** + transgene in the **ASI-4 mthmg1** context restored ascospore germination. This was done by crossing the **mthmg1** strain with different **ASI-4** transformants carrying either the 8F5 cosmid or the pBS5ma4.5 or pBSB5B (Fig. 1A) expressed at least at the same level as a resident **mthmg1** gene (Fig. 1B). Furthermore, the phenotypes associated with the **mthmg1** deletion allowed us to genetically localize the **mthmg1** gene. It appeared to map on linkage group I, very close to the centromere (second division segregation percentage of about 2), i.e., weakly linked to **ASI-3** (38).

We attempted to overexpress and deregulate the **mthmg1** gene by replacing its promoter region with the glyceraldehyde-3-phosphate dehydrogenase (**gpd**) promoter of *P. anserina* (39), a strong constitutive promoter. The gene fusion was cloned in pBC-Hygro, and the resulting plasmid was used in transformation experiments. Among the 15 transformants purified and studied from different transformation experiments, in at least 10 cases, introduction of **gpd:mthmg1** in an **mthmg1** + context led to major or minor alterations in germination, growth, fertility, and life span. The phenotypes observed are somewhat different from one transformant to another, but this can be correlated with the overexpression level (Fig. 1B). For example, one transformant showing only a threefold increase in **mthmg1** transcription (Fig. 1B, lane 7) displays only slightly altered germination while another with about a 10-fold increase (Fig. 1B, lane 8) displays a greatly altered phenotype similar to that of **mthmg1** strains. These differences in overexpression levels might be due to positional effects for different integration sites. Thus, overexpression (or constitutivity) of **mthmg1** seems to be detrimental to the fungus.

**ASI-1** + **mthmg1** strains show a particular mtDNA senescent pattern. The senescence syndrome (40), which occurs in all wild-type strains of *P. anserina*, is associated with major mtDNA rearrangements and the amplification of some regions as head-to-tail, circular, double-stranded molecules called senDNAs (see reference 7 for a review). senDNA$\alpha$ is systematically observed in large amounts in senescent cultures of wild-type strains (Fig. 2). senDNA$\beta$ is also frequently recovered (Fig. 2), as is senDNA$\gamma$. senDNA$\alpha$, senDNA$\beta$, and senDNA$\gamma$ are formed with monomers of variable sizes and termini, yielding amplified restriction fragments whose size varies between cultures (7). Analysis of mtDNA from several dying **mthmg1** strains showed no obvious amplification of senDNA$\alpha$ but showed more unusual senDNA which seemed to correspond to the $\gamma$ region (7) (Fig. 5A). Hybridization confirmed that only a small amount of senDNA$\alpha$ is amplified compared to a wild-type strain (Fig. 5B) and that some bands clearly seen in the ethidium bromide-stained gel indeed belong to the $\gamma$ region (Fig. 5C). In any case, the mtDNA rearrangements observed in **mthmg1** are clearly different from those observed in the wild-type strains.

**mthmg1** is essential in the **ASI-4** context. According to segregation analysis of the progeny of the crosses, **ASI-4 mthmg1** spores are formed but do not germinate. Sometimes, only one or two very short filaments emerge from the spore, but growth arrests very soon, even if the spore is transferred on growth medium and under a variety of germination temperatures. **mthmg1** is thus lethal in association with the **ASI-4** mutation. Introduction of an **mthmg1** + transgene in the **ASI-1 mthmg1** context restored ascospore germination. This was done by crossing the **mthmg1** strain with different **ASI-4** transformants carrying either the 8F5 cosmid or the pBS5ma4.5 or pBSB5B (Fig. 1A). To determine if the **ASI-4 mthmg1** lethality is specific for the **ASI-4** mutation, we have tested other **ASI** alleles, as well as other mutations altering cytoplasmic translation, and particularly those increasing translational accuracy (see Materials and Methods). In addition to **ASI-4** and **ASI-5**, three other **ASI** mutations are known (38). **mthmg1** is also lethal in an **ASI-5** context. In contrast to **ASI-4** and **ASI-5** mutants, **ASI-1** and **ASI-3** strains do not accumulate the mtDNA deletions when dying (M. Dequard-Chablat, unpublished results). Surprisingly, **mthmg1** is also lethal in association with the **ASI-1** mutation and sublethal...
with AS1-3. Some AS1-3 Δmthmg1 spores germinate but form very tiny thalli. After transfer on growth medium, the AS1-3 Δmthmg1 strains either stop growing or display a greatly altered phenotype with a low growth rate and a very spindly pigmented mycelium. All the other antisuppressor mutations tested, also altering translation (see Materials and Methods), did not prevent germination of Δmthmg1 spores. Thus, Δmthmg1 is lethal specifically in AS1 mutant contexts.

**mthmg1 is localized within the mitochondria.** Sequence analysis suggested that mthmg1 could be localized in the mitochondria and that it displayed some features of DNA-binding proteins. To determine its subcellular localization, mthmg1 was tagged at the COOH terminus with GFP (see Materials and Methods and Fig. 1A). To prevent expression variability due to integration sites, we tried to obtain several transformants, with the plasmid containing the construction integrated at different loci.

Transformants were screened in an AS1-5 rmp1-1 mthmg1 strain and selected for increased life span. Several transformants containing a second mthmg1 gene (fused with GFP) were studied which exhibited a clear in vivo fluorescence inside the mycelium. However, in most transformants, the construct was integrated very close to the resident mthmg1 gene, and it was not possible to introduce the construction in a Δmthmg1 context to confirm if the mthmg1-GFP fusion protein was fully active and able to complement the mthmg1 deletion. Finally, only three transformants were obtained for which the mthmg1-GFP copy was integrated independently from mthmg1 and could be associated with the Δmthmg1 null allele. Although considered functional since they were competent to increase the life span of an AS1-4 or AS1-5 rmp1-1 strain, they only partially complemented the Δmthmg1-associated phenotype. Northern blot analysis suggested that there are slightly fewer transcripts corresponding to the fusion gene than to the wild-type mthmg1 gene (data not shown). As shown in Fig. 6, in the filaments of strains bearing the mthmg1-GFP fusion, the fluorescence was localized to numerous small dots that overlapped with vitally DAPI-labeled mtDNA.

**DISCUSSION**

In this study, we describe mthmg1, the first gene identified as a double-copy suppressor of premature death syndrome in *P. anserina*. The premature death degenerative process was described several years ago (6) for certain AS1 mutants and was correlated with the accumulation of a deletion of one-third of the mtDNA. Two copies of mthmg1 only delay the accumulation of these defective molecules and have no obvious effect on wild-type behavior. Deletion of mthmg1 is lethal or sublethal in the AS1 mutant context, while it confers an altered phenotype in a wild-type background. The senescence of AS1 Δmthmg1 strains is accelerated and associated with a particular senDNA pattern without accumulation of senDNAa. As inferred from mapping data, mthmg1 does not correspond to previously identified genes whose mutations suppress premature death (14). Sequence analysis and subcellular localization of an mthmg1-GFP fusion suggest that mthmg1 is a mitochondrial protein associated with DNA.
mthmg1 may encode a functional homologue of the yeast ABF2. To date, only two mitochondrial proteins clearly belong to the family of HMG proteins: S. cerevisiae Abf2 (21) and metazoan transcription factor mtTFA (also called mtTF1 [36]). Abf2 and mtTFA each contain two HMG-1 boxes (4). Abf2 has been involved in mtDNA maintenance, but this is probably due to its pleiotropic role as a DNA-packaging protein (see references 15 and 33 for reviews). Human mtTFA plays an essential role in transcription, and its mouse homologue was also shown previously to participate in the maintenance of the mitochondrial genome (27, 48). The human protein (h-mtTFA) partly rescues the phenotypic defects of abf2 mutant yeast strains (37). According to PSI-Blast, the closest protein compared to mtHMG1 is h-mtTFA (23% identical aa, 43% similar aa). However, we note that, similarly to Abf2, the Podospora mtHMG1 protein lacks the highly charged tail essential for transcriptional activation activity in mtTFA (19). Some parallels between ABF2 and mthmg1 can be indeed drawn from the comparison of the effects of both deletion and overexpression of these genes. The disruption of yeast ABF2 yields viable cells on nonrespiratory substrates but leads to mtDNA instability (rapid loss of wild-type mtDNA on rich fermentable medium and no growth at high temperature on glycerol medium) (21, 32). P. anserina mthmg1− strains also display mitochondrial genome instability resulting in a short life span. Different and variable phenotypes were observed for transformants containing mthmg1 under the control of the strong Podospora gpd promoter. A strong phenotypic alteration is observed when the overexpression level is about 10-fold. In yeast, strong overexpression of ABF2 (≥10-fold) leads to a rapid loss of mtDNA while two to three copies of the gene slightly increase the amount of mtDNA (32, 50). If the Podospora mtHMG1 protein is a functional homologue of Abf2, we can suppose that its overproduction might also lead to loss of mtDNA and be deleterious for the fungus, while only one additional copy has no obvious effect in a wild-type background. Thus, mtHMG1 may perform functions similar to those of Abf2 in maintenance of the mitochondrial genome and, as an architectural protein, be involved, directly or by interaction with other proteins, in different steps of mtDNA metabolism such as recombination and/or distribution. It may be informative to attempt complementation of the Δmthmg1 strain with the yeast ABF2 gene and/or the human mtTFA cDNA.

mthmg1 encodes a novel HMG protein. The mthmg1 gene encodes a highly basic protein with no clear homologue, although one predicted protein from the N. crassa genome displays 30% identity with the P. anserina protein. If the two fungal proteins are functional homologues, they have clearly diverged, which could explain why no structural homologue from other organisms has been found in databases. However, from our results with mtHMG1-GFP fusion and sequence comparisons, it is probably a mitochondrial protein associated with nucleoids. A potential AT-hook motif in mtHMG1 is found, as in nuclear proteins that play important roles in chromatin structure and act as transcription factor cofactors (reference 9 and references therein), presumably to anchor these to particular DNA structures (minor grooves of AT-rich DNA regions and four-way junctions). At least 11 bacterial proteins also bear such a motif (SMART software). If this motif plays a role in mtHMG1 function, this protein will be the first example of an AT-hook-containing protein in mitochondria. Moreover, two mtHMG1 regions display similarities with HMG-1 boxes. HMG-1 proteins are DNA-binding proteins able to bend DNA and bind to distorted DNA structures; they thus appear to act as architectural facilitators in the assembly of nucleoprotein complexes (9). These observations suggest that mtHMG1 might play a role in mtDNA structure and even in recombination events. In any case, P. anserina mthmg1 is the first example of a protein combining the two DNA-binding domains, AT-hook motif and HMG-1 boxes, found till now in different subfamilies of high-mobility-group proteins.

FIG. 6. In vivo localization of an mtHMG1-GFP protein fusion in the mycelium. (A) GFP fluorescence of an AS1′ rmp1-1 strain containing the mtHMG1-GFP fusion. Punctate labeling is observed. Scale bar, 5 μm. (B) DAPI staining of the same sample. Large fluorescent spots correspond to nuclei, and small dots reveal mtDNA nucleoids.
above). In contrast, the absence of the protein results in a decreased life span with a particular senDNA pattern (accumulation of senDNAγ, at least) and limited senDNAα accumulation. An absence or reduced accumulation of senDNAα is usually correlated with an increase in life span. This was observed, for example, in the su12-1C1 and AS6-5 mutants impaired in the cytosolic translational apparatus (44), in the girscea mutant involved in the control of cellular copper homeostasis (8), and for the first premature death suppressor described, paTom70-1 (in the AS1+ context) (25). Disruption of the nuclear cox5 gene, encoding subunit V of the cytochrome c oxidase complex, also leads to an increase in longevity associated with accumulation of senDNAγ in more or less marked quantities (22). In contrast, mutations of the AS3 gene accelerate the senescence process with a spectacular accumulation of senDNAα (7). Short life spans with no accumulation of senDNAα were previously reported in two situations. The premature death phenotype of AS1-4/5 rmp1-1 is a paralytic death phenotype (11), and limited senDNA accumulation of senDNAγ (at least) and limited senDNAα accumulation. In contrast, the absence of the protein results in a particular senDNA pattern (accumulation of senDNAγ, at least) and limited senDNAα accumulation. Thus, the absence of mtHMG1 protein results in particular senDNA patterns different from those previously observed for short-lived strains, including those characteristic of premature death. Consequently, mtHMG1 may be involved, directly or indirectly, in the accumulation of senDNAα.

In a mutant AS1-4 context, we have shown that two copies of mthmg1 delay the accumulation of deletion molecules characteristic of premature death. Since mtHMG1 seems to be an architectural DNA-binding protein, it may either slow the accumulation of the deletion or be required for a proper and selective transmission of the wild-type mitochondrial genome. The absence of mtHMG1 protein in this context is lethal. We can assume that this is due to a very fast accumulation of defective genomes or, conversely, to an altered transmission of the wild-type molecules. The colthality of AS1 mutations and Δmthmg1 would thus be due to the lack of mtHMG1 in a context that impairs translation of other proteins also required for mtDNA maintenance. Below a critical functional threshold of these proteins, the defective genome(s) would overcome the functional genome. Further analyses will determine the functions of these proteins and provide a better understanding of the mechanism(s) that acts in accumulation of mtDNA deletions, not only in P. anserina but probably also in more complex eukaryotes.

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