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

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In the filamentous fungus 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 ASI-4 mutation, which alters a cytosolic ribosomal protein gene. The 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 ASI-4 context. The duplication of mthmg1 has no significant effect on the wild-type life span or on senDNA patterns. In an ASI-1 context, deletion of the mthmg1 gene alters germination, growth, and fertility and reduces the life span. The Δmthmg1 senescent strains display a particular senDNA pattern. This deletion is lethal in an ASI-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 mtHMG1-green fluorescent protein fusion, 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, mtHMG1 displays functional similarities with the 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 Saccharomyces cerevisiae (see reference 16 for a review). The filamentous fungus 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 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 ASI-4 or ASI-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 ASI-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 cosmold library from *P. anserina* genomic DNA and sought transfectants 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 *rpm1* gene is tightly linked to the *mut* locus and has two natural forms, *rpm1*-1, linked to *mat*–, and *rpm1*-2, linked to *mat*+ (previously called *rpm*– and *rpm*+, respectively) (13). This gene has been shown to play a key role in AS1-4 and AS1-5 longevity. The *rpm1*-2 allele (13) greatly delays the cessation of fungal growth compared to the *rpm1*-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 *rpm1*-1 strains in which premature death could be rapidly observed. Mutations increasing the life span of the AS1-4 *rpm1*-1 strain have been described previously (14). *rpm2* is one of these mutations: the life span of the AS1-4 *rpm1*-1 *rpm2* strain is over 90 cm, and the accumulation of the mtDNA deletion characteristic of premature death is not observed at the point of death.

Other mutations altering translation and characterized as informational anti-suppressors were used in this study: AS1-3 and AS1-7-2, probably altering modification enzymes of a ribosomal component; AS6-4, 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 (cornmeal 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 50 μg/liter and streptomycin (from Sigma) at 10 mg/liter, depending on the strain 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. 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 *rpm1*-2 strain (14). High-molecular-weight genomic DNA was digested with the *Sal* restriction enzyme. The fragments were ligated in the cosmold vector pMOcoxS, whose dominant selective marker is the bacterial hygromycin B resistance gene (*hph*) under the control of the Neor-pons *crassa* *cpc1* 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), *Escherichia coli* DH5α (24) was used for genomic library construction, and *CMV* (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 *Hae*III digestion and Southern blotting. Implants were removed at approximately 1 cm from the margin of dead mycelia. The specific probes used to reveal senescent death were then labeled with *Pfu* polymerase (Stratagene). Initially, a DNA fragment containing the GFP coding sequence was obtained by PCR from pEGFP-1 vector (Clontech) with an mtHMG1-GFP fusion primer (5'-GGCTCTTTTCTCTTCTTTCCCTATGGTAGCAAGGCGAGGAGG-3'), encompassing the 3' end of the mtHMG1 ORF without the stop codon, as well 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 Laloue) by two different methods (5'-RACE kit [Roche Molecular Biochemicals] and 5'-RACE System for Rapid Amplification of cDNA Ends, version 2.0 [Life Technologies]) according to the manufacturer's instructions and by using the three nested primers 8, R3, and R4 (see Fig. 1A and 3).

**Cloning procedures.** A search for two-copy suppressors of premature death was performed by selecting a cosmid(s) able to increase the AS1-4 *rpm1*-1 strain life span (more than 2 cm). However, the AS1-4 *rpm1*-1 strain dies too quickly to permit protoplast preparation. We thus constructed a heterokaryotic strain (AS1-4/AS1+*) in which the mutant nucleus is complemented by the dominant AS1+ allele. To distinguish protoplasts containing the two types of nuclei, the AS1+ nucleus was of opposite mating type and marked with the *lei*-1 mutation leading to leucine auxotrophy. After transformation, protoplasts isolated from the AS1-4 *lei*-* rpm1*-1 (mat–) AS1+ *lei*-* rpm1*-2 (mat+) strain were spread on RG medium containing hygromycin, but not leucine; thus, the only transformed AS1-4 nucleus-containing protoplasts were able to grow. However, some transformed protoplasts may contain both nuclei. In this case, the resulting strains will grow longer than AS1-4 *rpm1*-1 strains. But these *mat–/mat+* strains are self-fertile and can thus be discarded. In an initial experiment, 10 pools, each containing 96 cosmids, were used and about 2,000 transformants were analyzed. The SIB selection method (1) was used to identify a cosmid bearing a premature death suppressor (5SF). A 4.5-kb *Sma*I fragment was subcloned in *pBluescript* from the 8FS cosmid (yielding *pBSsma4.5*) (Fig. 1A) and further reduced to a 2.2-kb *Eco*RV fragment (*pSRV2*V2). Two other plasmids derived from *pBSsma4.5* were also used: *pBSBbg*, obtained after digestion with *Bam*HI (from the *pBluescript* polylinker), and *BgfII*, followed by religation excising a 1-kb fragment downstream of *mthmg1*, as well as *pBSchH*, obtained after digestion with *Hind*III and religation, eliminating a 1.7-kb fragment containing the promoter of *mthmg1* region and the start of the open reading frame (ORF) (Fig. 1A).

**Sequencing the *mthmg1* gene region.** The *Sma*I fragment was completely sequenced by using the ABI PRISM Ready Reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) with a 373A automatic sequencer (Applied Biosystems). Several subclones from *pBSsma4.5* 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 Appligene 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* (*Delta*mthmg1) was constructed by transposon *Tn7* in *pBSBbg* of the 366-bp *Pvu*AI-*Bcl* fragment (Fig. 1A) with *Pvu*II-*Bam*HI fragment containing the phleomycin resistance gene (*b1e*) from the *pPAb* plasmid (16). This plasmid was digested with *Nrl* and *Bgl*II, and the deletion-containing fragment was used (Fig. 1A). To circumvent the possibility of lethality of the *mthmg1* deletion, we transformed an AS1-4 *rpm1*-1 strain bearing an ectopic *mthmg1* copy previously obtained by transformation with the *pBSRVR2*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 *b1e* gene and two in the *mthmg1* sequence upstream of the *Nrl* site or downstream of the *Bgl*II site [Fig. 1A]), which should amplify a DNA fragment only if gene replacement occurred. For Southern hybridization, the DNA of the transform-ants was digested with *Nrl*, *Eco*RV, or *Eco*RI plus *Hind*III and hybridized with the 4.5-kb *Sma*I fragment as a probe. The fragments expected were obtained for eight primary transformants. Three were purified by crossing them with the wild-type strain to eliminate the ectopic copy linked to hygromycin resistance and analyze the phenotype of the deletion in AS1+ and AS1-4 contexts.

**Construction of GFP-tagged *mthmg1* and isolation of transgenic strains.** A green fluorescent protein (GFP) fusion to the COOH terminus of mtHMG1 was constructed by PCR with previously described *Pfu* polymerase and *Pics* primers. Initially, a DNA fragment containing the GFP coding sequence was obtained by PCR from pEGFP-1 vector (Clontech) with an mtHMG1-GFP fusion primer (5'-GGCTCTTTTCTCTTCTTTCCCTATGGTAGCAAGGCGAGGAGG-3'), encompassing the 3' end of the mtHMG1 ORF without the stop codon, as well as
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 AS1-4 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 AS1-4 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 pBC-Hygro as a cotransformation plasmid. A 2.2-kb fragment (Fig. 1A) was digested with the enzymes I site and the end of the terminator sequence, (ii) the pmtHMG1-GFP plasmid was constructed by ligation of the three following fragments: (i) the vector portion of pBSBBg digested with BglII and NcoI containing the 5' part of mthmg1 and the end of the terminator sequence, (ii) the BglII-BgGI fragment from pmtHMG1-GFP containing the end of the mthmg1 ORF fused with GFP coding sequence, and (iii) the above PCR fragment digested with BglII and NcoI. After sequencing, the final construction was subcloned in pBc-Hygro by using the NorI and SalI vector sites, yielding pBcmthmg1-GFP-GFP. This plasmid was introduced by transformation into BglII and NorI digested with the plasmid pBSBGl by using the Second, the mthmg1 terminator region was added at the end of the GFP coding sequence as follows. A fusion GFP-mthmg1 primer containing a BglII restriction site (5'-GCGTACAAATGTGGTATGGC-3') was used in conjunction with primer 10 to amplify a terminator fragment from pBSBGl (with Pfu DNA polymerase). The resultant pmtHMG1-GFP plasmid was constructed by ligation of the three following fragments: (i) the vector portion of pBSBBg digested with BglII and NcoI containing the 5' part of mthmg1 and the end of the terminator sequence, (ii) the BglII-BgGI fragment from pmtHMG1-GFP containing the end of the mthmg1 ORF fused with GFP coding sequence, and (iii) the above PCR fragment digested with BglII and NcoI. After sequencing, the final construction was subcloned in pBc-Hygro by using the NorI and SalI vector sites, yielding pBcmthmg1-GFP-GFP. This plasmid was introduced by transformation into mthmg1 or AS1-5 rmp1-1 protoplasts. Since the mthmg1 strain displays altered growth and a reduced life span and since these phenotypes are recessive, we used a heterokaryotic mthmg1-lac1 mut–nadh1-lac1 mut+ 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 longer than normal life span as does the AS1-4 rmp1-1 strain, thus permitting the preparation of homokaryotic protoplasts. Hygromycin-resistant transformants complemented for the growth defect of mthmg1 or selected for an increased life span of AS1-5 rmp1-1 were purified by cloning them with the wild-type strain and were submitted to cytological observation.

**mthmg1 overexpression.** A gene fusion with the gpd (glyceraldehyde-3-phospho dehydrogenase) promoter of *P. anserina* (39) was generated as follows. A gpd promoter-containing fragment was amplified from pGPAH1 (2) with PU and HOP2 primers, by Pfu DNA polymerase. A pgpd-mthmg1 fusion primer (5'-CTCCTTCTCACAACGCAACATCGCTCTCCTCCACTGG-3') was designed to amplify a fragment that included both the gpd promoter and the ORF in conjunction with the 5' portion of the mthmg1 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 purified fragment was digested with HindIII and inserted in pSReHL at the HindIII site. In the recombinant construct, the correct orientation of the PGPD fragment was confirmed by PCR directly on the colonies with primer 8 and PU and by plasmid extraction and digestion with EcoRI. The pgpd-mthmg1 plasmid was sequenced before use to ensure that no mutations were 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 Gel Band 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 0.015 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 estimated by densitometric scanning with Storm Imager and 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 mitochondrion-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 Princenton 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**

**Table 1. Effect of two copies of mthmg1 on life spans**

| Strain and transformant | Life span (cm) | mtDNA pattern |
|-------------------------|----------------|---------------|
| ASI+ mthmg1+           | 14.8 ± 1       | senDNAs       |
| ASI-4 mthmg1+          | 2 ± 0.4        | mtDNA deletions |
| ASI+ mthmg1+ (mthmg1+) b | 14.6 ± 2.8     | senDNAs       |
| ASI-4 mthmg1+ (mthmg1+) b | 11.6 ± 3.7     | senDNAs       |
| A                       | >110           | mtDNA deletions |
| B                       | >110           | mtDNA deletions |
| C                       | >90            | mtDNA deletions |
| ASI+ Δmthmg1            | 3.8 ± 1        | senDNAγ       |
| ASI-4 mthmg1+ (mthmg1+) b | ≈2.3           | Not tested |
| ASI-4 mthmg1+ (mthmg1+) b | 12.9 ± 3.7     | Not tested |

a 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 cosm id (A, B, and C), or the pBSmalt5.5 plasmid (F), both of which fully complemented the mthmg1 null allele (Δmthmg1). b Life spans were measured as described in Materials and Methods for at least 10 cultures. c 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. d Data were obtained with the ectopic copy of transformant F. e Mean of only three cultures.
abolish, the accumulation of the mtDNA deletions in an AS1-4 rnp1-1 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 EcoRV 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 EcoRV 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 Logan. Therefore, the mthmg1 gene is likely located in a mitochondrial compartment.

**FIG. 1.** (A) Partial restriction map of mthmg1 chromosomal region (4.5-kb SmaI fragment) and derived plasmid inserts. Only relevant restriction sites used in this study are shown. The black rectangle corresponds to the mthmg1 ORF with the intron represented by shading and the arrow indicating the transcription direction. Arrowheads indicate the localization, name, and direction of oligonucleotides used for PCR or sequencing. (B) Northern blot hybridizations of total RNA (about 30 μg) from wild-type (lanes 2, 4, and 6) and Δmthmg1 (lane 1) strains and purified transformants bearing mthmg1 ectopic copies carried by the 8F5 cosmid in the mthmg1 context (lane 3), pBSsma4.5 in the Δmthmg1 context (lane 5), and pgpd::mthmg1 (lanes 7 and 8, two integration sites leading to major or minor alteration phenotypes in the mthmg1 context). Northern blots were probed at the same time with mthmg1 (Sma4.5 fragment [this paper]) and AS1-specific probe (2, 20). AS1 was used for comparison of RNA amounts loaded in each well. Levels of mthmg1 transcript were normalized against AS1 mRNA levels in each lane. The mthmg1 gene in 8F5 copy is expressed at the same level as the resident gene while some plasmidic copies appear slightly overexpressed (twofold increase in a Δmthmg1 background, lane 5). In the case of pgpd::mthmg1, the transformant in lane 7 shows a slight increase (about 3-fold) in mthmg1 transcript, while that in lane 8 displays a clear overexpression (about 10-fold).
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-I 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).

**AS1** strains in which **mthmg1** is either absent or overexpressed display an altered phenotype. In order to examine the role of **mthmg1**, the resident gene was deleted as described in Materials and Methods. The \(PshI-BclI\) fragment was replaced with the phleomycin resistance gene (\(ble\)) as a selection marker (Fig. 1A). To eliminate the possibility of lethality of the null allele, we transformed an \(AS1-4\ mtp1-1\) strain bearing an
ectopic copy of \textit{mthmg1} (long life) and screened for \textit{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}.

\textbf{FIG. 3.} Nucleic acid and deduced protein sequences of the \textit{mthmg1} gene. The sequence of the \textit{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\textsuperscript{'} end of the messenger and to check the intron position are indicated with dashed arrows above the sequence. Vertical arrows indicate the major (\textit{s}) and minor (\textit{2}) 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{Sma}I 4.5-kb fragment has been submitted to EMBL (accession no. AJ316007).
ROLE OF TWO COPIES OF mthmg1 IN Podospora anserina

**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**/**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 pBSStma4.5 or pBSBGBg (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 three-fold 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 **Δmthmg1** 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-4** **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). senDNAα is systematically observed in large amounts in senescent cultures of wild-type strains (Fig. 2). senDNAβ is also frequently recovered (Fig. 2), as is senDNAγ. senDNAα and senDNAγ 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 **Δmthmg1** strains showed no obvious amplification of senDNAα but showed more unusual senDNA which seemed to correspond to the γ region (7) (Fig. 5A). Hybridization confirmed that only a small amount of senDNAα 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 γ region (Fig. 5C). In any case, the mtDNA rearrangements observed in **Δmthmg1** are clearly different from those observed in the wild-type strains.
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 phenotypes. 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 filamentous 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 senDNAs. 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.
spots correspond to nuclei, and small dots reveal mtDNA nucleoids. Scale bar, 5 µm. (B) DAPI staining of the same sample. Large fluorescent spots correspond to nuclei, and small dots reveal mtDNA nucleoids.

**FIG. 6.** In vivo localization of an mtHMG1-GFP protein fusion in the mycelium. (A) GFP fluorescence of an ASI+ rpm1-1 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.

**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.

**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* Δ*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 Δ*mthmg1* strain with the yeast *ABF2* gene and/or the human *mtTFA* cDNA.

**mtHMG1 and mtDNA.** In a wild-type *ASI*+ context, two copies of *mthmg1* have no evident effect on the life span and senDNA pattern of dying strains (see discussion of Abf2,
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 *girsea* 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 senDNA (7). Short life spans with no accumulation of senDNAα were previously reported in two situations. The premature death phenotype of *AS1-4/S rmp1-1* strains is associated with mtDNA deletions (6). The *paMDM10-1* mutant, screened as a premature death suppressor described, displays a relatively mild phenotype with a spectacular accumulation of senDNA (7). Short life spans with no accumulation of senDNAα were previously reported in two situations. The premature death phenotype of *AS1-4/S rmp1-1* strains is associated with mtDNA deletions (6). The *paMDM10-1* mutant, screened as a premature death suppressor described, displays a relatively mild phenotype with a spectacular accumulation of senDNA (7).

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 colothality of *AS1* mutations and Δ*mthmg1* 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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